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DESIGN, OPTIMIZATION AND *IN VIVO* EVALUATION OF NON-VIRAL VECTORS FOR GENE THERAPY ·APPLICATIONS IN OCULAR DISORDERS·
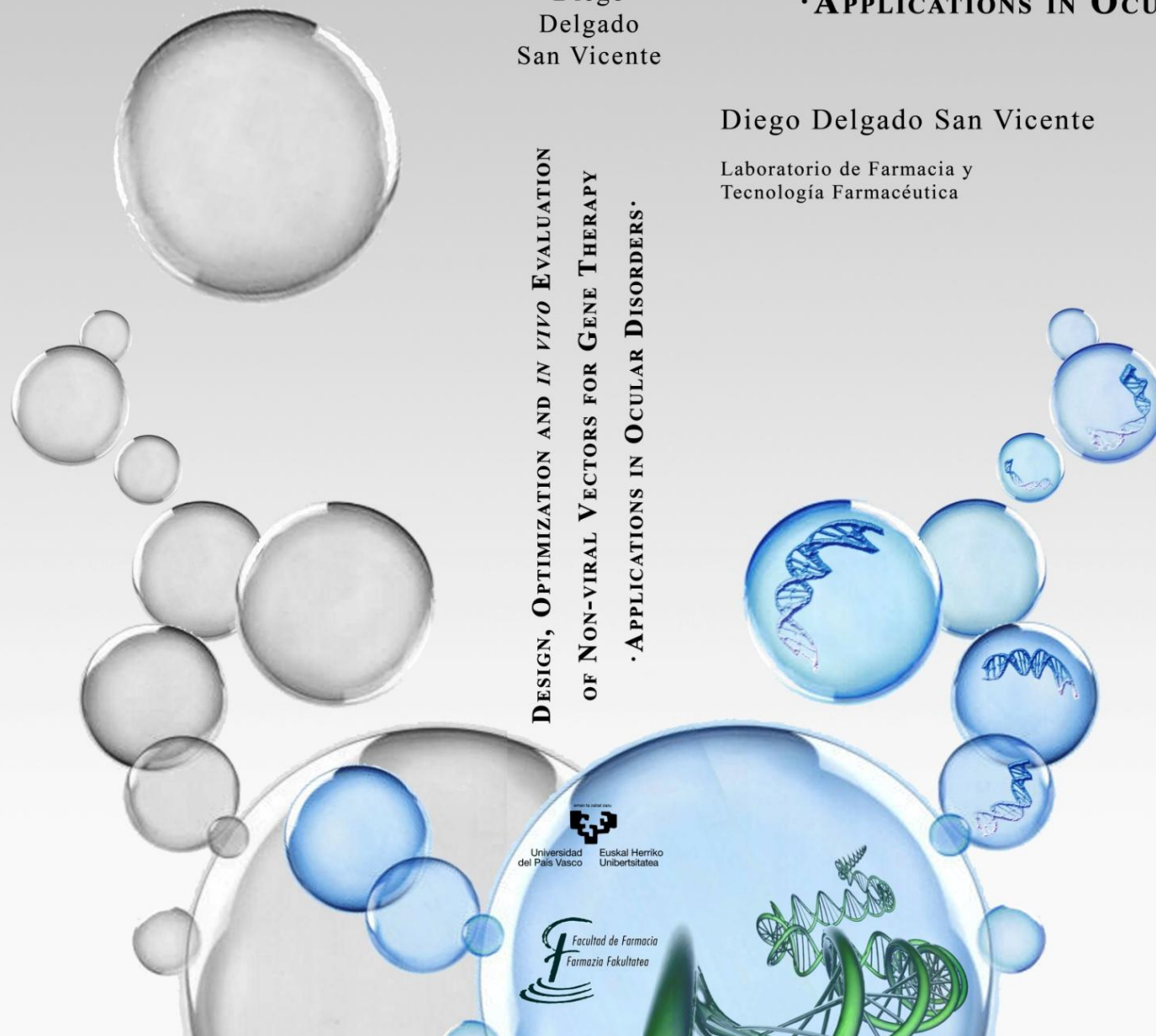
Diego Delgado San Vicente

Laboratorio de Farmacia y
Tecnología Farmacéutica

VITORIA-GASTEIZ 2011

Universidad
del País Vasco

Euskal Herriko
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Design, optimization and *in vivo* evaluation of non-viral vectors for gene therapy. Applications in ocular disorders.

Diego Delgado San Vicente

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"El agradecimiento es la memoria del corazón". No se me ocurre mejor forma para empezar que esta cita de Lao Tse, ya que a lo largo de estos años queda algo más que una recopilación de datos, análisis y conclusiones. Son gestos, palabras y sensaciones que deslizan ciertas personas y que sin ellas todo este trabajo no se hubiese llevado a cabo. O peor aún, hubiese sido un trabajo inútil. A estas personas dedico este trabajo (sabéis de sobra quienes sois):

Javier y Txelo, mis padres, no sólo habéis respetado mi silencio de una forma que yo no hubiese sido capaz, sino que lo habéis recompensado con ánimo, esfuerzo y sacrificio. Gracias a vosotros no me falta de nada y cualquier queja que saliese de mi boca sería un insulto. Ni siquiera mil tesis saldarían lo que hacéis por mí.

Alicia y Marian, mis directoras de Tesis, tanto con vuestra cabeza como con vuestro corazón habéis conseguido que este viaje haya llegado a buen puerto sin naufragar. Esta tesis demuestra que al final teníais razón. Y enhorabuena, os habéis doctorado en Paciencia. Seguid así, y no sólo hablo del plano profesional.

Ana, mi compañera, sabes que eres la tercera directora de esta tesis. Gracias por todo lo que me has enseñado y por todo lo que he aprendido de ti sin que te des cuenta. Eres un ejemplo a seguir y haría otra tesis si tú fueses mi directora. Sentiré envidia de tu primer doctorando, y de todos los siguientes.

Aritz y Paola, gracias a vosotros la continuidad de este trabajo está asegurada. Vuestra forma de ser y trabajar me ha ayudado enormemente los últimos años, y ha sido un placer enseñaros lo poco que sé (y me habéis enseñado mucho). Permitidme un último consejo: como he dicho antes, Alicia y Marian tienen razón (aunque duela).

A mis padres

El ojo que ves no es
ojo porque tú lo veas;
es ojo porque te ve.

- Antonio Machado –

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SUMMARY

Over the past few years and due to biotechnology development, gene therapy has emerged as a promising therapeutic tool because of the use of exogenous nucleic acids as active ingredients. These nucleic acids, i.e. Deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), can be delivered into the organism in order to modulate the expression of proteins, which are modified in particular diseases.

These acquired or genetic diseases include pathologies such as cancer, AIDS, neurological disorders or autoimmune diseases. Ocular gene therapy is one of the most investigated fields and its progress lies, among other things, in the easy accessibility into the eye and the convenient methods for its evaluation. Thus, several research groups use gene therapy as alternative to treat diseases such as retinitis pigmentosa, macular degeneration and X-linked juvenile retinoschisis.

The vast majority of these studies are carried out using viral vectors to deliver genetic material, as they have proved to be the most effective to date. Nevertheless, the safety-related risks that involve their use have triggered the development of non-viral vectors, and the improvement of their efficacy has become a key challenge in gene therapy. In order to design non-viral vectors, an understanding of the extra and intracellular barriers conditioning the transfection process is needed: interaction and subsequent passage through cellular membrane, intracellular trafficking, resistance

against degradation, genetic material release, passage through nuclear membrane and finally, expression of the protein codified by the plasmid that vector transports.

In the present work hybrid gene delivery systems have been developed and evaluated. These systems, based on solid lipid nanoparticles (SLN), also include compounds of different nature to favor the transfection process by mans of several mechanisms. Formulations were characterized in terms of size, surface charge, protection capacity against DNAses and plasmid release from the nanoparticle. Furthermore, "in vitro" transfection capacity, cellular uptake, endocytosis mechanisms and intracellular trafficking were studied by means of confocal laser microscopy and flow cytometry. These processes were carried out over two cell lines (ARPE-19 and HEK-293) and were related to transfection capacity of the formulations developed.

Firstly, the influence of a peptide, protamine, in the transfection capacity of SLN was evaluated. The presence of protamine in SLN increased the "in vitro" transfection capacity in ARPE-19 cells, with respect to SLN without protamine, but it caused a decrease in HEK-293 cells. It was observed that this different behavior is conditioned by the cellular uptake mechanism of nanoparticles, which depends on cell line as well as vector composition.

Next, a polysaccharide, dextran, was included in the formulation. When lipid nanoparticles were prepared with protamine and the dextran, a high transfection capacity was achieved in ARPE-19 cells with the plasmid that codifies green fluorescent protein (EGFP) as well as the one that codifies retinoschisin, a protein related to X-linked juvenile retinoschisis.

Once the vector was optimized by means of "in vitro" studies, the "in vivo" evaluation was conducted. For that purpose, a vector prepared with SLN, protamine, dextran and the plasmid that codified EGFP was administrated by different ocular routes in rats. After intravitreal injection, retina ganglion cells were mainly transfected, and when administrated by subretinal injection, retinal pigment epithelium cells as well as photoreceptors were the most transfected cells. After topical application, the vector was also able to transfect corneal cells.

Finally, transfection capacity after intravenous administration into mice was also evaluated, detecting green fluorescent protein in liver, spleen and lung; the expression was maintained for at least seven days.

En los últimos años y debido al desarrollo de la biotecnología, la terapia génica ha emergido como una prometedora herramienta terapéutica gracias al uso de los ácidos nucleicos exógenos como principios activos. Estos ácidos nucleicos, como el ácido desoxirribonucleico (ADN) o el ácido ribonucleico (ARN), pueden ser vehiculizados y administrados para modificar la expresión de proteínas que están alteradas en determinadas enfermedades.

Entre las enfermedades que pueden ser potencialmente tratadas mediante terapia génica se incluyen tanto enfermedades genéticas como adquiridas, por ejemplo, cáncer, SIDA, neuropatologías o enfermedades autoinmunes. La terapia génica ocular es una de las áreas que más ha sido investigada y que más ha evolucionado, en parte por las facilidades que presenta el ojo tanto desde el punto de vista de la administración como de su evaluación. Así, existen varias líneas de investigación en las que se contempla la terapia génica como una alternativa para tratar enfermedades como la retinosis pigmentaria, la degeneración macular o la retinosquisis juvenil ligada al sexo.

La mayoría de los estudios sobre terapia génica utilizan vectores virales como sistemas de vehiculización del material genético, ya que han resultado ser hasta el momento los más eficaces. Sin embargo, las importantes desventajas desde el punto de vista de la seguridad de los vectores virales, han hecho aumentar el protagonismo de los vectores no virales. Debido a su menor capacidad de transfección, el principal reto a la hora de desarrollar este tipo de sistemas es incrementar su eficacia. El diseño de un vector no viral implica un conocimiento adecuado de los pasos limitantes, tanto

a nivel extraceluar como intracelular que condicionan el proceso de transfección: interacción y posterior paso a través de la membrana celular, difusión intracelular, resistencia a la degradación, liberación del material genético, paso a través de la membrana nuclear y posterior expresión de la proteína que codifica el plásmido que transporta, proceso conocido como transfección.

En este trabajo se han desarrollado y evaluado sistemas no virales de transporte y liberación de ADN. Estos sistemas, a base de nanopartículas sólidas lipídicas (SLN), también incluyen otros componentes de diferente naturaleza que por diversos mecanismos, pueden favorecer la transfección. Las formulaciones elaboradas se caracterizaron en términos de tamaño de partícula, carga superficial, capacidad de protección frente a DNAsas y capacidad de liberación del plásmido de la nanopartícula. También se ha estudiado, mediante técnicas de microscopía confocal y citometría de flujo, la capacidad de transfección "in vitro" en dos líneas celulares (ARPE-19 y HEK-293), la capacidad de ser internalizadas a nivel celular, el mecanismo implicado en su captación y su disposición intracelular. Estos procesos fueron relacionados con la capacidad de transfección de las diferentes formulaciones desarrolladas.

En primer lugar, se estudió la influencia de un péptido, la protamina, en la capacidad de transfección de las SLN. La presencia de protamina incrementó la capacidad de transfección "in vitro" de estas en células ARPE-19, pero la disminuyó en células HEK-293. Se observó que este diferente comportamiento está condicionado por el mecanismo de internalización celular de las nanopartículas, que depende tanto de la línea celular como de la composición del vector.

Posteriormente se incluyó un polisacárido, dextrano, en las formulaciones. Cuando las nanopartículas lipídicas se prepararon con protamina y dextrano, se obtuvo una alta eficacia de transfección en células ARPE-19, tanto con el plásmido que codifica la proteína verde fluorescente (EGFP), como con el que codifica la retinosquisina, proteína alterada en la retinosquisis juvenil ligada al sexo.

Una vez optimizado el vector mediante estudios "in vitro", se procedió a su evaluación "in vivo". Para ello, el vector preparado con las SLN, la protamina, el dextrano y el plásmido que codifica la EGFP se administró por vía ocular a ratas utilizando diferentes vías de administración. Cuando el vector se administró por vía intravítrea, se transfectaron mayoritariamente células ganglionares, y cuando se administró por vía subretinina, células del epitelio pigmentario de la retina y fotorreceptores. Tras la administración por vía tópica, se transfectaron células corneales.

Finalmente, se evaluó también su capacidad de transfección tras administración sistémica por vía endovenosa a ratones, detectándose la proteína verde fluorescente en hígado, bazo y pulmón, cuya expresión se mantuvo al menos durante siete días.

INTRODUCTION
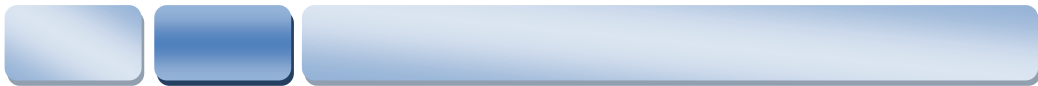
1. TERAPIA GÉNICA OCULAR

Según la Agencia Europea del Medicamento (EMA), la terapia génica está englobada dentro del grupo conocido como terapias avanzadas. Estos medicamentos son de uso humano y están basados en genes (terapia génica), células (terapia celular) o tejidos (terapia tisular). Las terapias avanzadas ofrecen nuevas estrategias para abordar enfermedades que hasta el momento carecen de tratamientos eficaces.

Figura 1. *Terapias avanzadas.* Situación de la terapia génica respecto al resto de tratamientos.

El Reglamento del Parlamento Europeo (CE Nº 1394/2007) define a los medicamentos de terapia génica como:

"medicamento biológico con las características siguientes:

- *incluye un principio activo que contiene un ácido nucleico recombinante, o está constituido por él, utilizado en seres humanos, o administrado a los mismos, con objeto de regular, reparar, sustituir, añadir o eliminar una secuencia génica;*
- *su efecto terapéutico, profiláctico o diagnóstico depende directamente de la secuencia del ácido nucleico recombinante que contenga, o del producto de la expresión genética de dicha secuencia.*

Los medicamentos de terapia génica no incluyen las vacunas contra enfermedades infecciosas".

Así pues, el objetivo final de la terapia génica es introducir en la célula diana material genético con el fin de modular la expresión de determinadas proteínas que se encuentran alteradas, revirtiendo así el trastorno biológico que provoca la alteración de esas proteínas.[1]

Para que una enfermedad sea candidata a ser tratada mediante terapia génica, debe conocerse el papel del gen en la fisiopatología de esa enfermedad.[2] También es necesario caracterizar adecuadamente el sistema de administración del gen o vector, para controlar su localización en el organismo y la duración de la expresión génica. La Figura 2 representa un esquema de la terapia génica.
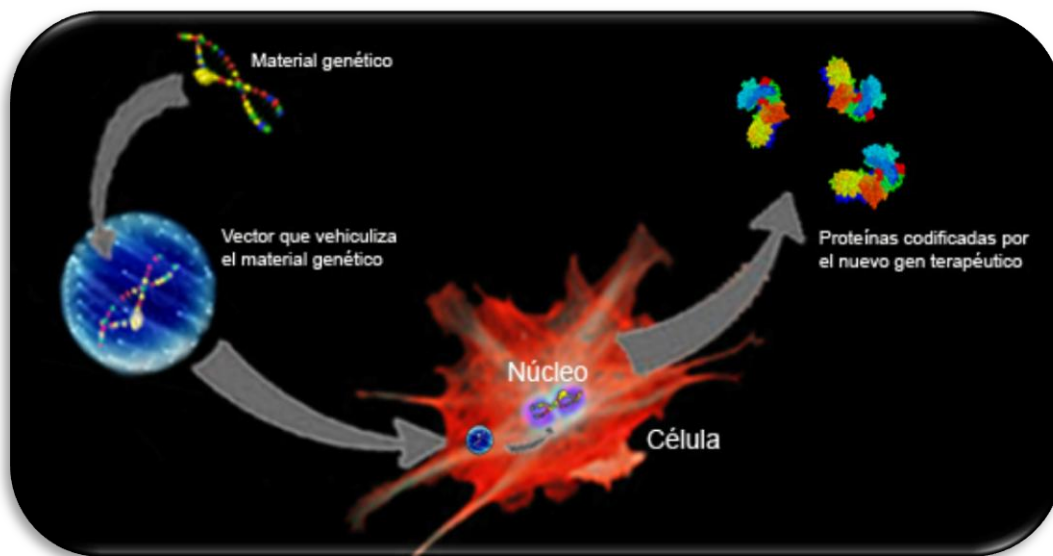
Figura 2. *Terapia génica.* El objetivo es introducir ADN o ARN en células diana a través de un vector y liberar el material genético; de esta forma se modifica la expresión de las proteínas relacionadas con la enfermedad.

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[1] Novelli G, Gruenert DC. Genome medicine: gene therapy for the millennium. Pharmacogenomics. 2002;3:15-18.
[2] Flotte TR. Gene therapy: the first two decades and the current state-of-the-art. J Cell Physiol. 2007;213:301-305.

Existe un amplio número de enfermedades que pueden llegar a tratarse con terapia génica; desde enfermedades monogénicas como la fibrosis quística, hasta enfermedades más complejas como el cáncer.[3] Un grupo de afecciones que está despertando un gran interés es el relacionado con los problemas oculares. El ojo presenta una serie de ventajas (Figura 3) para la aplicación de este tipo de terapia debido a:

- Su anatomía bien definida.
- Su fácil accesibilidad, evitando de esta forma la administración sistémica y las barreras que se han de superar para llegar al lugar de acción.
- La reducida dimensión del tejido ocular y la baja difusión a la circulación sistémica, lo que permite utilizar bajas dosis.
- La posibilidad de utilizar métodos no invasivos y de poder comprobar directamente la recuperación mediante la evolución de la función visual.
- El privilegio inmunológico del que goza este órgano.[4]
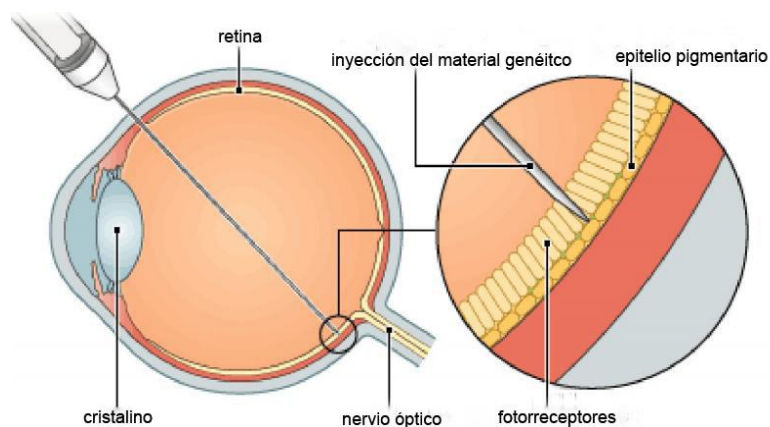
Figura 3. *Terapia génica ocular.* El ojo es un órgano prometedor para la terapia génica debido a las características que presenta.

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[3] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Terapia Génica: Concepto, Sistemas de Administración y Aplicaciones. Industria Farmacéutica. Nº 128. Julio/Agosto 2006.

[4] Naik R, Mukhopadhyay A, Ganguli M. Gene delivery to the retina: focus on non-viral approaches. Drug Discov Today. 2009;14:306-315.

1.1. Formas de administración del material genético

La terapia génica puede aplicarse "ex vivo" extrayendo las células del paciente, haciéndolas crecer en cultivo, administrando el material genético "in vitro" e introduciéndolas de nuevo en el paciente; o "in vivo", método mediante el cual el material genético se introduce directamente en las células del paciente sin tener que extraerlas previamente; sin embargo, el grado de control sobre todo el proceso es mucho menor que con la terapia "ex vivo". La Figura 4 muestra un esquema en el que se indican ambos procesos.
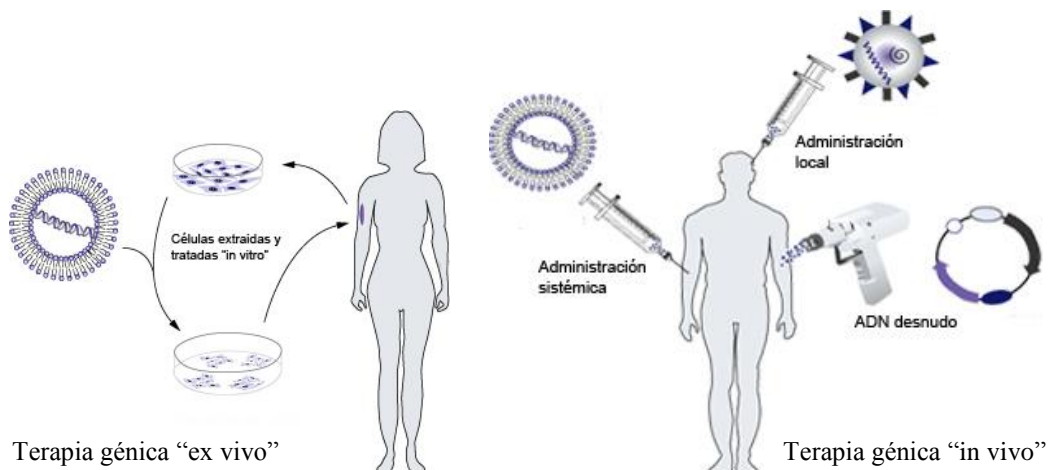
Figura 4. *Terapia génica "ex vivo" e "in vivo".*
En la terapia génica "ex vivo", las células son extraídas del paciente y transfectadas "in vitro".
En la terapia génica "in vivo", se introduce el ADN directamente en el paciente.

Desde el punto de vista clínico y farmacéutico, es más aceptable el uso de la terapia génica "in vivo", ya que permite la utilización de las vías de administración habituales y es mucho más sencilla que la modalidad "ex vivo".

La introducción en el núcleo celular de moléculas de ADN se conoce como transfección celular, y para que tenga lugar, el material genético se puede administrar utilizando diversos métodos.

Una de las posibilidades de la terapia génica "in vivo" consiste en administrar ADN desnudo (*naked DNA*); es decir, administrar ADN de doble hélice que previamente se ha multiplicado en algún tipo de bacteria. Mediante esta técnica se ha conseguido transfección a nivel local cuando se administra ADN en determinados tejidos. Por ejemplo, la administración intramuscular de ADN desnudo tiene gran interés en el desarrollo de vacunas basadas en terapia génica.[5]

En este sentido, la vía intradérmica ha de tenerse en cuenta para la administración de ADN. La Figura 5 recoge los niveles de transfección a nivel local obtenidos en un estudio[6] realizado con ratones a los que se les administró mediante inyección intradérmica el gen desnudo que codifica para la luciferasa formulado en diferentes vehículos: agua, solución de dextrosa al 5% y solución PBS.
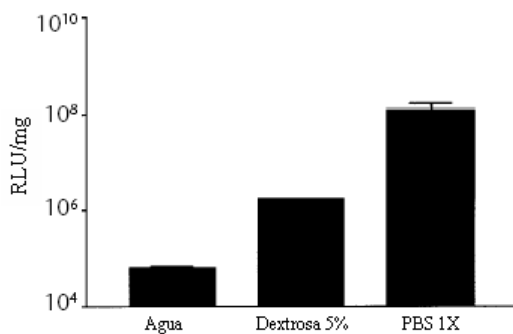
Figura 5. Transfección obtenida mediante la administración intradérmica de ADN desnudo en diferentes vehículos. Se midió la actividad de la enzima luciferasa codificada por el ADN administrado. Los resultados se presentan como unidades de luz relativas por mg de proteína total (RLU/mg).

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[5] Brown MD, Schätzlein AG, Uchegbu IF. Gene delivery with synthetic (non viral) carriers. Int J Pharm. 2001;229:1-21
[6] Chesnoy S, Huang L. Enhanced cutaneous gene delivery following intradermal injectoin of naked DNA in a high ionic strenght solution. Mol Ther. 2002;5:57-62.

Uno de los inconvenientes del uso del ADN desnudo es su degradación enzimática. Para evitar esta degradación, se emplean sistemas de administración que protegen al plásmido, facilitan su captación celular y lo liberan en el citoplasma. Hay dos grupos de sistemas en función de la naturaleza del vector: sistemas virales y sistemas no virales.

1.2. Sistemas virales

Compuestos por virus modificados genéticamente (Figura 6), con ellos se consigue que el gen se exprese en las células diana sin que tenga lugar la replicación del virus. Permiten obtener transfecciones muy altas; sin embargo, conllevan problemas debido a su inmunogenicidad y a su potencial oncogénico; además, el tamaño del ADN que pueden transportar es muy limitado. Su potencial

Figura 6. *Vector viral.*

oncogénico depende de la capacidad que tenga el virus para insertar el ADN en el genoma de la célula huésped. Esta inserción provoca altos niveles de transfección a largo plazo, pero puede inducir mutagénesis y dar lugar a la aparición de células tumorales. La inmunogenicidad, común a todos los virus, puede reducirse en parte eliminando las proteínas víricas, que son las responsables de este problema. Los virus más utilizados en terapia génica son los virus adeno-asociados, adenovirus, alfavirus, virus herpes simplex, retrovirus y lentivirus. La elección del virus más adecuado dependerá de la enfermedad a tratar y por tanto, de las células que se quieren transfectar. El Cuadro 1 recoge un resumen de vectores virales aplicados en terapia génica ocular.[7]
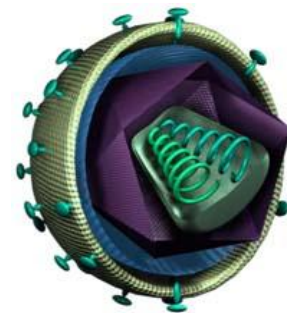
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[7] Colella P, Cotugno G, Auricchio A. Ocular gene therapy: current progress and future prospects. Trends Mol Med. 2009;15:23-21.

VECTORES VIRALES PARA TERAPIA GÉNICA OCULAR

Los genes virales son parcial o completamente borrados del genoma del vector viral y sustituidos por la combinación transgénica deseada.

Lentivirus

Su estructura permite generar vectores híbridos con envolturas de glicoproteínas, como con la glicoproteína G del virus de la estomatitis (VSV-G), que confiera buena respuesta y estabilidad. Pueden transportar una secuencia de hasta 8kb.

Adenovirus

Obtenidos por la eliminación parcial del genoma viral; sin embargo, los genes virales remanentes provocan respuesta inmune. Por eso se han generado nuevos "Ad-vectors" eliminando todo su genoma. Pueden encerrar secuencias de 36kb.

Virus Adeno-asociados (AAVs)

Son pequeños virus (AAVs) no patogénicos con una sola cadena de ADN. Se obtienen por la eliminación de todas las secuencias virales.

La imagen muestra la expresión de la proteína verde fluorescente en células del epitelio pigmentario y fotorreceptores tras la administración de un vector adeno-asociado (rAAV2/1 CMV-GFP) mediante inyección subretiniana.
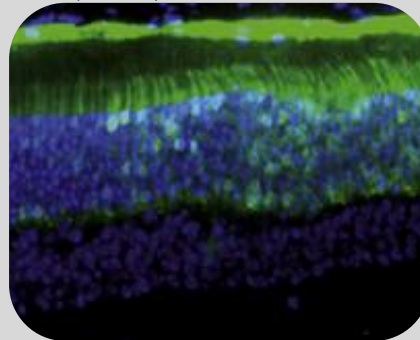
Enfermedad	Vector	Transgen
Retinoblastoma	Adenovirus	Herpes thymidine kinase
Degeneración macular	Adenovirus	Pigment Epithelium factor
Amaurosis congénita de Leber	Adeno-asociado	RPE65
Amaurosis congénita de Leber	Adeno-asociado	RPE65
Amaurosis congénita de Leber	Adeno-asociado	RPE65

Ensayos clínicos de terapia génica ocular

Cuadro 1. *Vectores virales utilizados en terapia génica ocular.* [7]

1.3. Sistemas no virales

En estos sistemas, el plásmido se multiplica en bacterias y se combina con diferentes componentes: polímeros, péptidos, lípidos o mezcla de ellos.

A pesar de que su eficacia de transfección no es tan alta como con los sistemas virales, se producen fácilmente a gran escala con bajo coste. Además, tienen capacidad para transportar moléculas de ADN de mayor tamaño y presentan mayor seguridad al no ser ni inmunogénicos, ni oncogénicos, ya que el ADN que portan no se inserta en el genoma de la célula. Debido a que la principal limitación es la baja transfección, muchos grupos de investigación estamos centrados en el desarrollo de estrategias que mejoren la capacidad de transfección de estos sistemas.

Estos vectores pueden administrarse en el ojo mediante varios tipos de inyección (Cuadro 2). La elección de la vía depende de las células que se desean tratar y de las características del vector.[7]

Inyección intravítrea: para tratar glaucomas y neovascularizaciones de la retina interna.

Inyección subretiniana: para el tratamiento de mutaciones y degeneraciones del epitelio pigmentario de la retina y fotorreceptores.

Inyección intracameral: para moléculas antiinflamatorias que reducen la inflamación después del trasplante de córnea.

Inyección subconjuntival: para el uso de proteínas antiangiogénicas a través del espacio periocular en neovascularizaciones.

Cuadro 2. *Tipos de inyección intraocular utilizados en terapia génica .[7]*

Sistemas poliméricos

En terapia génica se recurre muchas veces a los polímeros policatiónicos, que son aquellos que presentan carga positiva a pH fisiológico. De ese modo, se produce una interacción electrostática entre el polímero, con carga positiva, y el ADN, que tiene carga negativa, y se forma el correspondiente complejo, llamado *"poliplex"*.
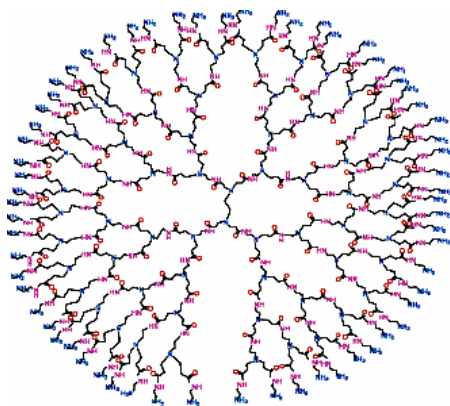
Figura 7. *Dendrímeros como vectores no virales.*
Dendrímero de poly(admidoamina).

Algunos de estos polímeros catiónicos utilizados en terapia génica son: poli (L-Lisina) (PLL)[8], polietilenimina (PEI)[9], quitosano[10], dendrímeros[11] (Figura 7) y ciclodextrinas.[12] En ocasiones, también se recurre a polímeros no catiónicos, como el ácido poli-láctico co-glicólico (PLGA)[13], que aunque no condensa el ADN tanto como los polímeros catiónicos, también lo protege de la degradación enzimática. Su toxicidad y su dificultad para producirlos a gran escala son sus principales inconvenientes.

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[8] Liu G, Molas M, Grossmann GA, Pasumarthy M, Perales JC, Cooper MJ, et al. Biological properties of poly-L-lysine-DNA complexes generated by cooperative binding of the polycation. J Biol Chem. 2001;276:34379-34387.

[9] Zhang C, Yadava P, Hughes J. Polyethylenimine strategies for plasmid delivery to brain-derived cells. Methods. 2004;33:144-150.

[10] Kim TH, Park IK, Nah JW, Choi YJ, Cho CS. Galactosylated chitosan/DNA nanoparticles prepared using water-soluble chitosan as a gene carrier. Biomaterials. 2004;25:3783-3792.

[11] Ramaswamy C, Sakthivel T, Wilderspin AF, Florence A. Dendriplexes and their characterisation. Int J Pharm. 2003;254:17-21.

[12] Cryan SA, Holohan A, Donohue R, Darcy R, O'Driscoll CM. Cell transfection with polycationic cyclodextrin vectors. Eur J Pharm Sci. 2004;21:625-633.
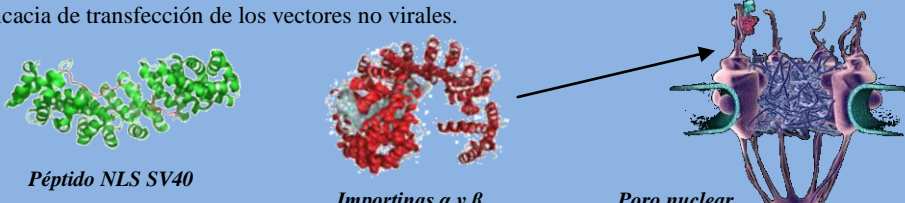
[13] Ravi Kumar RM, Bakowsky U, Lehr CM. Preparation and characterization of cationic PLGA nanospheres as DNA carriers. Biomaterials. 2004;25:1771-1777.

Sistemas peptídicos

Los péptidos catiónicos que se emplean en terapia génica contienen en su estructura aminoácidos con carga positiva, como son la histidina, la lisina o la arginina, a los que se puede unir el ADN. Algunos autores han propuesto el uso de péptidos sintéticos con secuencias activas similares a las proteínas víricas,[14] o de péptidos que facilitan la entrada en la célula denominados péptidos de penetración celular (CPPs).[15] Sin embargo, por sí solos no producen suficiente transfección y hay que combinarlos con componentes lipídicos[16] o poliméricos.[17] También destacan los péptidos con secuencias de localización nuclear (Cuadro 3).[18]

Péptidos con secuencias de localización nuclear (NLS)

En las células con baja capacidad de división celular, el transporte de moléculas hacia el núcleo tiene lugar principalmente través de los poros de la membrana nuclear. Las moléculas de pequeño tamaño pueden difundir libremente a través del poro nuclear, pero las de mayor tamaño requieren un transporte activo. Este transporte es activado por la interacción de determinadas moléculas con las importinas del núcleo (α y β). Las moléculas que interaccionan con estas importinas son las secuencias de localización nuclear (NLS), en las que destacan los aminoácidos lisina y arginina. Los péptidos con NLS se unen al ADN exógeno y mejoran la entrada de éste al núcleo incrementándose la eficacia de transfección de los vectores no virales.

Péptido NLS SV40   *Importinas α y β*   *Poro nuclear*

La interacción de los péptidos NLS con las importinas α y β permite el transporte a través del poro nuclear.

Cuadro 3. *Péptidos con secuencias de localización nuclear.*[18]

[14] Wyman TB, Nicol F, Zelphati O, Scaria PV, Plank C, Szoka FC. Design, synthesis and characterization of a cationic peptide that binds to nucleic acids and permeabilizes bilayers. Biochemistry. 1997;36:3008-3017.

[15] Chen S, Zhuo RX, Chen SX. Enhanced gene transfection with addition of a cell-penetrating peptide in substrate-mediated gene delivery. J Gene Med. 2010;12:705-713.

[16] Tokunaga M, Hazemoto N, Yotsuyanagi T. Effect of oligopeptides on gene expression: comparison of DNA/peptide and DNA/peptide/liposome complexes. Int J Pharm. 2004;269:71-80.

[17] Lee H, Jeong JH, Park TG. A new gene delivery formulation of polyethylenimine/DNA complexes coated with PEG conjugated fusogenic peptide. J Controll Rel. 2001;76:183-192.

[18] Duvashani-Eshet M, Keren H, Oz S, Radzishevsky IS, Mor A, Machluf M. Effect of peptides bearing nuclear localization signals on therapeutic ultrasound mediated gene delivery. J Gene Medicine. 2008;10:1150-1159.

Sistemas lipídicos

Los componentes generales de estas formulaciones son los lípidos, neutros o catiónicos. Estos últimos confieren carga positiva al sistema para que interaccione electrostáticamente con el ADN formando así el llamado *"lipoplex"*, el cual entra en la célula mediante endocitosis (Cuadro 4)[19]. Estos sistemas son bien tolerados por el organismo y pueden prepararse sin necesidad de usar solventes orgánicos.

Entrada del "lipoplex" en la célula

El complejo lípido-ADN se une con la membrana plasmática y es internalizado mediante endocitosis. Después de la endocitosis, parte del ADN es degradado debido a la fusión del endosoma con el lisosoma. Sin embargo, otra parte del ADN es liberado desde el endosoma al citoplasma, pudiendo así llegar al núcleo de la célula.

Existen dos rutas endocíticas principales mediante las cuales se internalizan los vectores no-virales: *vía clathrinas*, y *vía caveolas*.
La transfección puede incrementarse dirigiendo el vector hacia un determinado camino o usando secuencias de localización nuclear que dirijan el ADN al núcleo.

Cuadro 4. *Mecanismo de endocitosis de los vectores lipídicos para terapia génica.* [18]

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[19] Wasungu L, Hoekstra D. Cationic lipids, lipoplexes and intracellular delivery of genes. J Control Release. 2006;116:255-264.

Existen dos tipos de vectores lipídicos que se han venido aplicando en terapia génica: liposomas[20] y nanopartículas sólidas lipídicas (SLN).[21] Los liposomas son vesículas esféricas compuestas por una o más bicapas lipídicas, que rodean un núcleo acuoso. Las SLNs son partículas esféricas del rango de los nanómetros. Están formadas por una matriz sólida lipídica y son capaces de proteger moléculas como el ADN de la degradación enzimática y de controlar su liberación de esas moléculas. Además se pueden liofilizar fácilmente para favorecer su conservación. Las fotografías de la figura 8 fueron obtenidas mediante cryo-TEM (criomicroscopía de transmisión electrónica) por Kuntsche et al.[22]
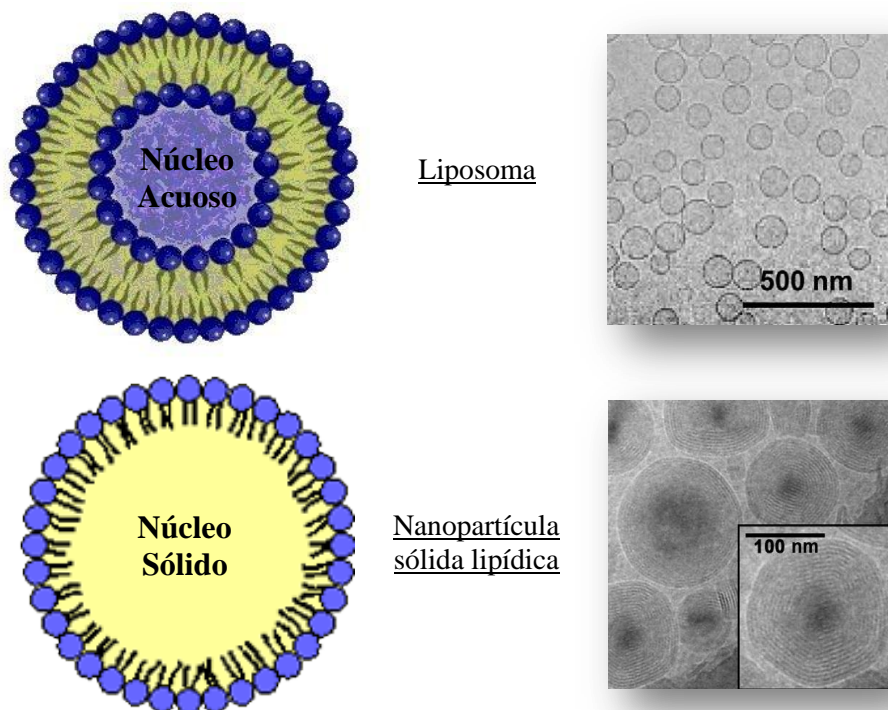
Figura 8. *Diferencias entre un liposoma y una nanopartícula sólida lipídica (SLN).*

[20] Tabatt K, Kneuer C, Sameti M, Olbrich C, Müller RH, Lehr CM et al. Transfection with different colloidal systems: comparison of solid lipid nanoparticles and liposomes. J Control Release. 2004;97:321-332.

[21] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles: formulation fators affecting cell transfecion capacity. Int Journal Pharm. 2007;339:261-268.

[22] Kuntsche J, Horst JC, Bunjes H. Cryogenic transmission electron micrscopy (cryo-TEM) for studying the morphology of coloidal drug delivery systems. Int J Pharm. 2011. *In press*

1.3.1. Sistemas no virales en la terapia génica ocular

Debido a los malos resultados obtenidos en la transfección de células oculares utilizando ADN desnudo,[23] el desarrollo de vectores no-virales se ha convertido en una parte fundamental de la terapia génica ocular.

Unos de los vectores más empleados han sido los liposomas, que mediante la combinación de diferentes lípidos como el colesterol, el DOPE (1,2-Dioleoyl-3-phosphatidylethanolamine) o el DOTAP (1,2-Dioleoyl-3-trimethyl ammonium propane), han conseguido transfectar células de la retina tanto "in vitro"[24] como "in vivo".[25] Para intentar aumentar la eficacia y el tiempo de expresión génica producida por estos vectores, los liposomas se han combinado con péptidos con secuencias de localización nuclear como la protamina,[26] o con proteínas virales como las del virus hemaglutinante de Japón; estos últimos se emplearon para tratar neovascuralización en la coroides inducida en ratas.[27]

La capacidad de las nanopartículas basadas en polímeros para escapar de la degradación endo-lisosomal ha hecho que también se empleen estos tipo de sistemas poliméricos en la terapia génica ocular. Por ejemplo, vectores de polyethylenimina (PEI) y ácido poli(láctico-coglicólico) (PLGA) han sido capaces de transfectar células

[23] Cai X, Nash Z, Conley SM, Fliesler SJ, Cooper MJ, Naash MI. A partial strucutral and functional rescue of a retitnitis pigmentosa model with compacted DNA nanoparticles. PLoS One. 2009;4:e5290.

[24] Peeters L, Sanders NN, Jones A, Demeester J, De Smedt SC.Post-pegylated lipoplexes are promising vehicles for gene delivery in RPE cells. J Control Release. 2007;121:208-217.

[25] Kawakami S, Harada A, Sakanaka K, Nishida K, Nakamura J, Sakaeda T et al. In vivo gene trsnsfection via intravitreal injection of cationic liposome/plasmid DNA complexes in rabbits. Int J Pharm. 2004;278:255-262.

[26] Mannermaa E, Rönkkö S, Ruponen M, Reinisalo M, Urtti A. Long-lasting secretion of transgene product from differentiated and filter-grown retinal pigment epithelial cells after non viral gene transfer. Curr Eye Res. 2005;30:345-353.

[27] Otsuji T, Ogata N, Takakashi K, Matsushima M, Uyama M, Kaneda Y. In vivo gene transfer into choroidal neovascularization by the HVJ liposome method. Graefes Arch Clin Exp Ophtalmol. 2000;238:191-199.

ganglionares de retina[28] y del epitelio pigmentario[29], respectivamente. El uso de dendrímeros también ha dado sus frutos al transfectar secuencias de oligodesoxinucleótidos tanto "in vitro"[30] como "in vivo".[31]

Entre los sistemas no virales peptídicos destacan las nanopartículas CK30-PEG10k, basadas en péptidos de lisina y cisteína conjugadas con poli-etilenglicol.[32] Estos vectores consiguieron transfectar diferentes tejidos oculares tras su administración por vía intravítrea y subretinal en ratones (Figura 9).[33] Además también se han empleado péptidos de penetración celular para facilitar la entrada en las células, consiguiendo transfectar células del epitelio pigmentario, fotorreceptores y células ganglionares de retina. Estos péptidos compuestos principalmente por los aminoácidos glicina, arginina, lisina y alanina son denominados POD (*Peptides for Ocular Delivery).*[34]
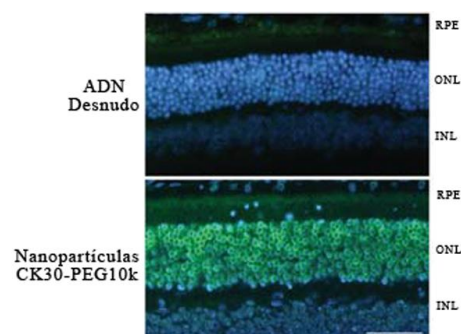
Figura 9. *Transfección obtenida en la retina de ratón.* RPE: epitelio pigmentario de retina. ONL: capa de células nucleadas externa. INL: capa de células nucleadas interna. Marcaje azul: núcleos celulares. Marcaje verde: expresión de la proteína verde fluorescente.[33]

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[28] Liao HW, Yau KW. In vivo gene delivery in the retina using polyethylenimine. Biotechniques. 2007;42:285-288.

[29] Bejjani RA, BenEzra D, Cohen H, Rieger J, Andrieu C, Jeanny JC, et al. Nanoparticles for gene delivery to retinal pigment epithelial cells. Mol Vis. 2005;11:124-132.

[30] Marano RJ, Wimmer N, Kearns PS, Thomas BG, Toth I, Brankov M et al. Inhibition of in vitro VEGF expression and choroidal neovascularization by synthetic dedndrimer peptide mediated delivery of a sense oligonucleotide. Exp Eye Res. 2004;79:525-535.

[31] Marano RJ, Toth I, Wimmer N, Brankov M, Rakoczy PE. Dendrimer delivery of an anti-VEGF oligonucleotide into the eye: a long-term study into inhibition of laser-induced CNV, distribution, uptake and toxicity. Gene Ther. 2005;12:1544-1550.

[32] Liu G, Li D, Pasumarthy MK, Kowalczyk TH, Gedeon CR, Hyatt SL, et al. Nanoparticles of compacted DNA transfect postmitotic cells. J Biol Chem. 2003. 278:32578-325786.

[33] Farjo R, Skaggs J, Quiambao AB, Cooper MJ, Naash MI. Efficient non-viral ocular gene transfer with compacted DNA nanoparticles. PLoS One. 2006;1:e38.

[34] Johnson LN, Cashman SM, Kumar-Singh R. Cell-penetrating peptide for enhanced delivery of nucleic acids and drugs to ocular tissues including retina and cornea. Mol Ther. 2008;16:107-114.

2. *LA TERAPIA GÉNICA EN LAS DISTROFIAS DE RETINA*

Las distrofias de retina son un heterogéneo conjunto de desórdenes que, aunque a veces adquiridos, se producen principalmente por causas genéticas asociadas a la retina; representan el problema visual hereditario más frecuente, teniendo una prevalencia de 1 por cada 3000 personas.[35]

2.1. La retina

La retina es la capa interior de la pared ocular y tapiza la túnica vascular (coroides) sin adherirse a ella. Es la parte funcional del ojo, convirtiendo la energía luminosa en energía nerviosa. Estructuralmente es una túnica fina y transparente formada por tejido nervioso y en la que se pueden distinguir varias capas. La más externa es el epitelio pigmentario, que está en contacto con la coroides. Las demás capas, que son de tejido nervioso, están formadas principalmente por tres tipos de células muy especializadas: fotorreceptores (conos y bastones), células bipolares y células ganglionares. Además existen células gliales que desempeñan funciones de soporte, nutricionales o de respuesta inmune como las células de Müller y los astrocitos. En la Figura 10 se recoge un esquema del globo ocular y la retina.

Figura 10. *Anatomía de la retina.* Situación de la retina debajo de la coroides y las diferentes capas que la forman.

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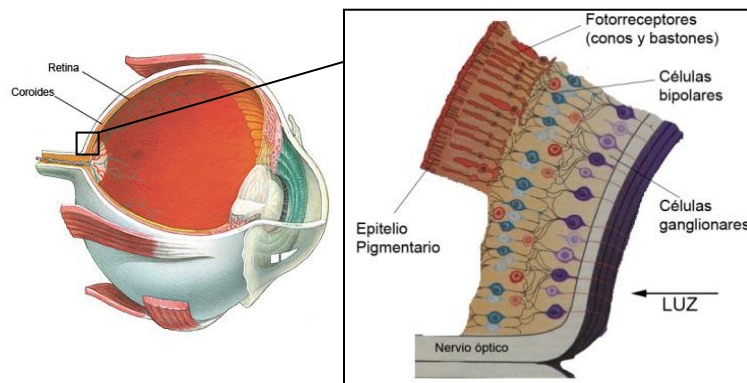
[35] Bessant DAR, Ali RR, Bhattacharya SS. Molecular genetics and prospects for therapy of the inherited retinal dystrophies. Curr Opin Genet Dev. 2001;11:307-316.

Epitelio pigmentario

Esta monocapa de células pigmentadas que forma parte de la barrera sangre/retina tiene su membrana apical en contacto con el segmento externo de los fotorreceptores. Esencial en la función visual, puede llevar a la degeneración de la retina y a la ceguera si falla una de las siguientes funciones:

- Participar en el recambio de los discos de las membranas del segmento externo de los fotorreceptores, fagocitándolos y reconstruyéndolos.
- Intervenir en el ciclo visual (Cuadro 5).
- Sintetizar melanina para absorber el exceso de luz que no es capturada por los fotorreceptores.
- Aportar nutrientes como glucosa, retinol y ácidos grasos desde la sangre a los fotorreceptores.
- Transportar iones, agua y productos metabólicos finales desde el espacio subretiniano a la sangre.
- Secretar factores de crecimiento que favorecen la integridad estructural, e inmunosupresores para mantener el privilegio inmunológico ocular.[36]

Epitelio pigmentario y ciclo visual

El 11-cis-retinal es activado por la luz en los fotorreceptores, lo que desencadena una serie de etapas que conducen a la transformación de la luz en señales eléctricas y el envío al cerebro.

Al ser activado, el 11-cis-retinal se transforma en all-trans-retinal, el cual debe volver a reisomerizarse en 11-cis-retinal. Este proceso no puede llevarse a cabo en los fotorreceptores y son las células del epitelio pigmentario las encargadas de esta función.
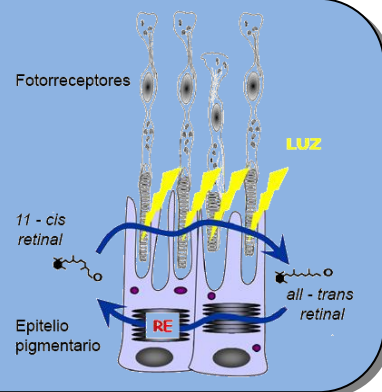
Cuadro 5. *Ciclo visual.* [36]

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[36] Strauss O. The Retinal Pigment Epithelium in Visual Function. Physiol Rev. 2005;85:845-881.

Se encuentran a continuación del epitelio pigmentario de la retina proyectando sus segmentos. Se diferencian dos partes: segmento externo, donde se realiza la transducción (Cuadro 6)[37], y segmento interno, donde se llevan a cabo procesos metabólicos. Se diferencian dos tipos de fotorreceptores:

- Los *bastones,* cuyo pigmento es la rodopsina; se encargan de la visión nocturna y en blanco y negro. Predominan en la periferia de la retina.
- Los *conos,* cuyos pigmentos son las fotopsinas y participan en la visión diurna y del color. Predominan en el centro de la retina.

La zona de mayor densidad de fotorreceptores se encuentra en la parte central de la retina (fóvea). A medida que nos alejamos hacia la periferia disminuye su número.

Fototransducción

La luz que penetra en el ojo es detectada por las neuronas especializadas de la retina: los fotorreceptores. Los fotones de la luz son absorbidos por la rodopsina en los bastones y por las opsinas en los conos **(a)** (para esto hace falta el 11-cis-retinal: Cuadro 5). La excitación de estos pigmentos activa la transductina por su unión al GTP **(b)**. Al unirse el GTP a la transductina, se activa la enzima fosfodiesterasa (PDE) que convierte el cGMP en GMP **(c)**. Así, disminuyen los niveles de cGMP, cerrando el canal iónico; la reducción del flujo de iones a través de la membrana produce la señal eléctrica que es transmitida al cerebro. La desactivación de este proceso se produce por la interacción de las proteínas RGS9 y R9AP, que convierten el GTP de la transductina en GDP, desactivando esta proteína y liberando fosfato (P) **(d)**.

Cuadro 6. *Fototransducción.* En este proceso la información visual captada por las células es convertida en señal eléctrica y enviada al cerebro.[37]

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[37] Blumer KJ. The need for speed. Nature. 2004;427:20-21.

Células bipolares y ganglionares

Los fotorreceptores contactan con las células bipolares, las primeras con las que establecen la sinapsis. Esta vía sensitiva sigue hacia el cerebro a través de las células ganglionares, cuyos axones constituyen el nervio óptico y junto con los vasos retinianos forman el punto ciego de la retina (Figura 11).
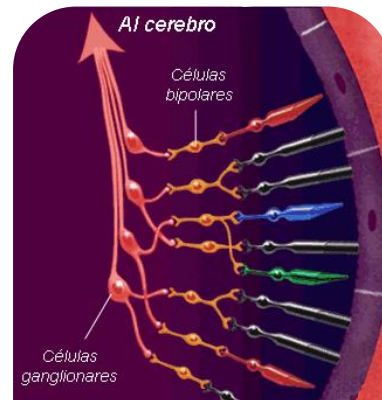
Figura 11. De la retina al cerebro.

2.2. Barreras a superar en terapia génica de retina

El principal problema de la terapia génica en la retina es la baja concentración de dosis que se alcanza en el lugar de aplicación, debido a la gran protección y estancamiento que posee este órgano.

Si los vectores se administran por vía intravenosa, han de atravesar la barrera hematoacuosa, en la parte anterior del ojo, y la barrera hematorretiniana, en la parte posterior. Esta barrera está formada por los capilares de la retina y las células del epitelio pigmentario, impidiendo el paso de moléculas desde el torrente sanguíneo a la retina; de esta forma la cantidad de material genético que alcanza su objetivo es mínima. Además, a través la barrera hematorretiniana también se eliminan del ojo sustancias altamente lipófilas como pueden ser algunos vectores no virales.[38]

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[38] Hornof M, Toropainen E, Urtti A. Cell culture models of the ocular barriers. Eur J Pharm Biopharm. 2005;60:207-225.

Si se opta por la administración tópica en la superficie ocular del ojo, la dosis que llega a la retina también es muy limitada. La penetración a través de la córnea es muy dificultosa, y parte de los sistemas de administración pueden ser absorbidos por la circulación sanguínea conjuntival y nasal.[39] Además, el paso por el humor vítreo es un gran impedimento.

El humor vítreo es una estructura gelificada de fibras de colágeno surcada por filamentos de proteoglicanos, proteínas estructurales y compuestos del suero. Los dos principales componentes estructurales son el colágeno y el ácido hialurónico. En los espacios interfibrilares se disponen proteoglicanos con sulfato de condroitina y sulfato de heparina. De esta forma se constituyen los glicosaminoglicanos, que hacen que el humor vítreo pueda interaccionar con vectores liposómicos y poliméricos, inmovilizándolos y desestabilizándolos.[40]

Este paso a través del humor vítreo también condiciona la administración intravítrea. La baja vida media intravitreal de las móleculas de ADN hace que sea necesario el uso de repetidas administraciones, lo que puede dañar los tejidos y producir desprendimiento de retina.

Por último, la administración subretiniana permite incrementar el tiempo de contacto de los vectores con las capas de la retina, pero el área en la que se produce el efecto está limitada sólo al punto de inyección. Además, se pueden producir lesiones en el epitelio pigmentario de la retina.[41]

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[39] del Amo EM, Urtti A. Current and future ophtalmic drgu delivery systems. A shift to the posterior segment. Drug Discov Today. 2008;13:135-43.

[40] Peeters L, Sanders NN, Braeckmans K, Boussery K, Van de Voorde J, Demeester J, et al. Vitreous: a barrier to nonviral ocular gene therapy. Invest Ophtalmol Vis Sci. 2005;46:3553-3561.

[41] Masuda I, Matsuo T, Yasuda T, Matsuo N. Gene transfer with liposomes to the intraocular tissues by different routes of administration. Invest Ophtalmol Vis Sci. 1996;37:1914-1920.

Figura 12.
Barreras de la terapia génica aplicada en la retina. Imagen modificada de Naik et al.[4]
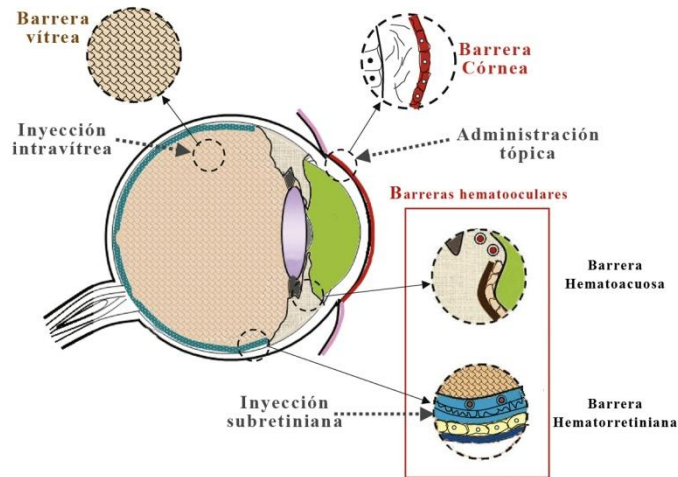
2.3. Aplicación de la terapia génica en el tratamiento de distrofias de retina

Las distrofias de retina son enfermedades de una gran complejidad y heterogeneidad genética. Durante los últimos años se ha procedido al mapeo e identificación de los genes responsables y de las proteínas que codifican dichos genes, distribuidos por todo el genoma. Su carácter hereditario, la evolución progresiva y no tener en el momento actual un tratamiento ni paliativo ni curativo, hacen que se produzca una pérdida total o parcial de la visión. Se clasifican según algunos de los siguientes criterios: síntomas clínicos, fenotipo electroretinográfico y patrón hereditario. De modo general se pueden dividir en:

- *formas periféricas*, en las que se afectan inicial y predominantemente los bastones de la retina y cuyo principal ejemplo es la retinosis pigmentaria.
- *formas centrales*, con degeneración de los conos desde su inicio pudiendo afectarse secundariamente o no los bastones (distrofias conos → bastones). Aquí se encuentran la enfermedad de Stargardt, distrofias maculares, etc.[42]

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[42] Ayuso García C. Retinopatías hereditarias en España". Foro complutense. Fundación General UCM ONCE. 2001.

En el Cuadro 7 se recoge un esquema con algunos genes implicados en enfermedades hereditarias de retina, la degeneración que causa su mal funcionamiento y la zona de la retina donde realizan su función:[35]

Epitelio
Pigmentario de Retina

Gen OA1
Albinismo
Cromosoma X (Locus Xp22.3)
Herencia ligada al Cromosoma X

Gen CHM
Coroideremia
Cromosoma X (Locus Xq21.2)
Herencia ligada al Cromosoma X

CICLO VISUAL
(Cuadro 5)

Gen RPE65
Amaurosis Congénita de Leber
Cromosoma 1 (Locus 1p31)
Herencia Autosómica Recesiva

Gen ABCA4
Enfermedad de Stargardt
Cromosoma 1 (Locus 1p21)
Herencia Autosómica Recesiva

Gen RS1
Retinosquisis Juvenil Ligada al Sexo
Cromosoma X (Locus Xp22.13)
Herencia ligada al Cromosoma X

Gen RHO
Retinosis Pigmentaria
Cromosoma 3 (Locus 3q22.1)
Herencia Autosómica Dominante

Fotorreceptores
(Fototransducción Cuadro 6)

Cuadro 7. *Genética y enfermedades hereditarias de retina.*
Retnet.(http://www.sph.uth.tmc.edu/Retnet/disease.htm).

2.3.1. Albinismo

Esta enfermedad congénita es producto de un defecto en la síntesis o distribución del pigmento melanina. Sintetizada a partir del aminoácido tirosina en unos orgánulos llamados melanosomas, la melanina es responsable de la pigmentación de la piel, el pelo y los ojos. Existen diferentes formas de albinismo que se dan como consecuencia de mutaciones de genes involucrados en la producción y acumulación de este pigmento. Cuando el albinismo afecta a la piel, al pelo y a los ojos se habla de albinismo oculocutáneo (*oculocutaneous albinism, OCA)*; el albinismo que afecta exclusivamente a los ojos se denomina albinismo ocular (*ocular albinism, OA)*. Dentro de esta última forma de albinismo, el más frecuente es el ocular tipo I (OA1) y se transmite con un patrón de herencia recesivo ligado al cromosoma X. De esta forma son los hombres los que presentan los principales síntomas de esta enfermedad, que cursa con una reducción visual aguda y que representa el mayor problema de esta afección; además, se acompaña de nistagmo (oscilación espasmódica del globo ocular), estrabismo y fotofobia.

El gen responsable del albinismo ocular tipo I, el gen *OA1* (GPR143), se expresa únicamente en los melanocitos del epitelio pigmentario de la retina y de la piel y su funcionamiento está explicado en el Cuadro 8. Varios estudios han demostrado que la ausencia del producto del gen OA1 en el epitelio pigmentario de la retina afecta a la función de los fotorreceptores. La terapia génica puede ser una opción a tener en cuenta en el tratamiento de esta enfermedad. En un estudio llevado a cabo en un modelo de ratón con albinismo ocular tipo I, se administró el gen OA1 en un vector viral elaborado con virus adenoasociados. Tras la administración, se observó una mejoría tanto en la morfología como en la funcionalidad de la retina incrementándose el número de melanosomas.
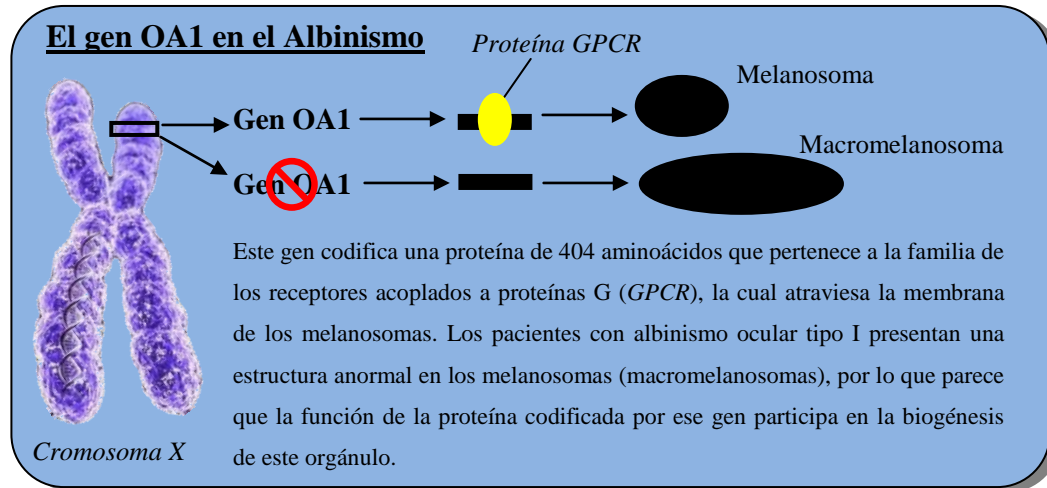
Cuadro 8. *Función del gen OA1 en el albinismo.*

En la Figura 13 se observan los cambios en los melanosomas antes y después del tratamiento. Estos resultados justifican las buenas expectativas de la terapia génica como potencial tratamiento del albinismo y otras enfermedades relacionadas.[43]
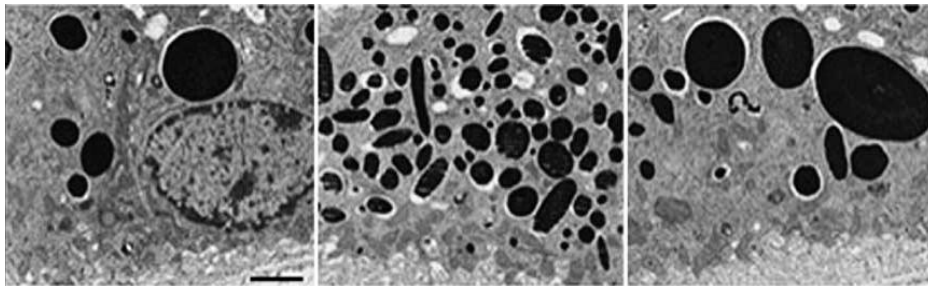
Figura 13. *Terapia génica aplicada al albinismo.* La foto de la izquierda muestra los macromelanosomas propios del albinismo. Las otras dos fotos muestran la retina tratada con adenovirus asociados que portan el gen OA1: aunque persisten los macromelanosomas (derecha), se observa claramente la presencia de nuevos melanosomas de tamaño normal (centro).

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[43] Surace EM, Domenico L, Cortese K, Cotugno G, Di Vicino U, Ventura C, et al. Amelioration of both functional and morphological abnormalities in the retina of a mouse Model of ocular albinism following AAV-mediated gene transfer. Mol Ther. 2005;12:652-658.

2.3.2. Amaurosis Congénita de Leber

La Amaurosis Congénita de Leber (LCA), de herencia autosómica recesiva, es la forma más temprana y severa de todas las distrofias retinianas responsables de déficit congénito de la visión. La pérdida de visión en estos pacientes suele manifestarse ya a los pocos meses de vida y progresa hasta una ceguera casi total en la adolescencia. Además, hay ceguera nocturna, disminución casi por completo del electroretinograma (ERG) en bastones y ausencia de respuesta en los conos.

Entre el 10% y el 16% de casos de LCA en Europa y América del Norte se debe a mutaciones en el gen RPE65. Este gen codifica una proteína de 65 kDa implicada en el ciclo del 11-*cis*-retinal y es específica del epitelio pigmentario de la retina (Cuadro 9).

El gen RPE65 en la Amaurosis Congénita de Leber

all-*trans*-retinal → 11-*cis*-retinal
Gen RPE65

all-*trans*-retinal → 11-*cis*-retinal
Gen RPE65

Epitelio Pigmentario

El gen RPE65 codifica una proteína de 65 kDa implicada en la isomerización del all-*trans*-retinal (proveniente de los fotorreceptores) a su forma fotoactiva 11-*cis*-retinal, proceso que tiene lugar en el epitelio pigmentario. De esta forma cuando RPE65 no es funcional no se sintetiza 11-*cis*-retinal, lo que lleva a un fallo en el ciclo visual (Cuadro 5) y a la imposibilidad de que se desencadene la fototransducción (Cuadro 6).

Cromosoma 1

Cuadro 9. *Función del gen RPE65 en la Amaurosis congénita de Leber.*

La deficiencia de este gen en ratones muestra acumulación de all-*trans*-retinal, niveles indetectables de rodopsina, disfunción de los bastones, inclusiones en el epitelio pigmentario y lenta degeneración de la retina (Figura 14).[44]
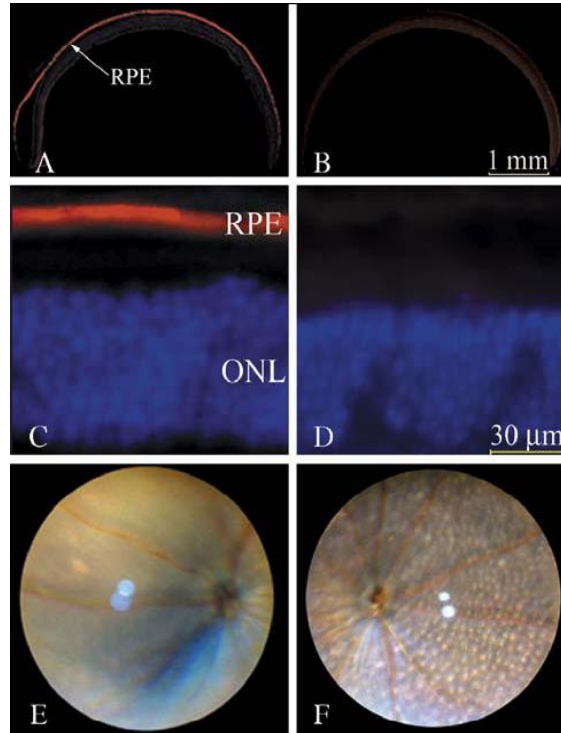
Figura 14. *Terapia génica en ratones con LCA*. Las fotos A y C muestran la expresión de la proteína codificada por el gen RPE65, introducido con vectores virales. Las fotos B y D corresponden al ojo no tratado. En la figura E se ve en el fondo del ojo la recuperación de la retina tratada, no así en la fotografía F que presenta la retina del ojo no tratado.

La terapia génica supone una esperanzadora alternativa para el tratamiento de la LCA. En 2001 se llevó a cabo un estudio en el cual se transfectaron células en cultivo del epitelio pigmentario de retina de perros con disfunción en el gen RPE65[45]. Los autores del trabajo emplearon adenovirus asociados como vectores virales portadores del gen RPE65 (AAV-RPE65). Una vez realizada la transfección, se inyectaron esas células intraocularmente a tres perros deficientes en el gen RPE65. En un ojo, la inyección fue intravítrea y en el otro subretiniana. Este segundo tipo de

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[44] Pang JJ, Chang B, Kumar A, Nusinowitz S, Noorwez SM, Li J, et al. Gene therapy restores vision-dependent behavior as well as retinal structure and function in a mouse model of RPE65 Leber Congenital Amaurosis. Mol Ther. 2006;13(3):565-572.

[45] Acland GM, Aguirre GD. Gene therapy restores vision in a canine model of childhood blindness. Nat. Genet. 2001;28:92–95.

inyección restauró parcialmente la funcionalidad de la retina y se demostró tanto cuantitativa (electroretinograma, pupilometría) como cualitativamente (estudios de conducta a los 4 meses); además, la expresión del gen normal se mantenía a los 99 días. La inyección intravítrea no mostró mejoría alguna. Estos resultados aparecen en la Tabla 1.

Animal	Edad	Ojo	Inyección	Efecto
BR29	132	D	SR	+
		I	IV	-
BR33	124	D	SR	+
		I	IV	-
BR47	108	D	SR	+
		I	IV	-
BR46	108	D	NI	-
		I	NI	-

Tabla 1. *Resultados de un estudio en el que se administraron vectores virales a perros con deficiencia de RPE65.* Los ojos de los perros tratados mediante inyección subretiniana mostraron respuesta positiva en el electroretinograma, lo que no sucedió con la inyección intravítrea. La respuesta fue medida a los 95 días. Edad: días; D: derecho; I: Izquierdo; SR: subretinal; IV: intravítrea; NI: no inyectado.

En un estudio posterior, Benniceli y cols.[46] administraron un vector viral elaborado con adenovirus modificados a perros que presentaban la enfermedad. El tratamiento produjo una mejoría en la visión de los perros y mediante inmunohistoquímica se comprobó la transducción del epitelio pigmentario de retina. Además, los estudios histopatológicos revelaron una mínima toxicidad.

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[46] Bennicelli A, Wright, Komaromy A, Jacobs JB, Hauk B, Zelenaia O, et al. Reversal of blindness in animal model of Leber Congenital Amaurosis using optmized AAV2-mediated gene transfer. Mol Ther. 2008;16:458-465.

Tras los buenos resultados obtenidos en los modelos animales, se iniciaron tres ensayos clínicos en 2007 (NCT00481546, NCT00516477, NCT00643747, clinicaltrials.gov.). Se siguió la evolución de un total de 18 pacientes con amaurosis congénita de Leber a los que se administraron vectores virales adeno-asociados cargados con el gen RPE65 (AAV2-RPE65) en el espacio subretinal del ojo más afectado de cada paciente. Tras el tratamiento con terapia génica hubo importantes mejoras en la función visual de los pacientes, la cual se produjo en un corto periodo de tiempo (unos pocos días y semanas) y persistió hasta un año y medio.[47] Además, tales mejoras fueron especialmente importantes en los niños tratados (8, 9 y 10 años), quienes consiguieron atravesar un circuito de obstáculos incluso con poca luz. Esto demuestra que si el tratamiento se realiza a edad temprana evitando la evolución de la enfermedad, los resultados son mucho más prometedores.[48]
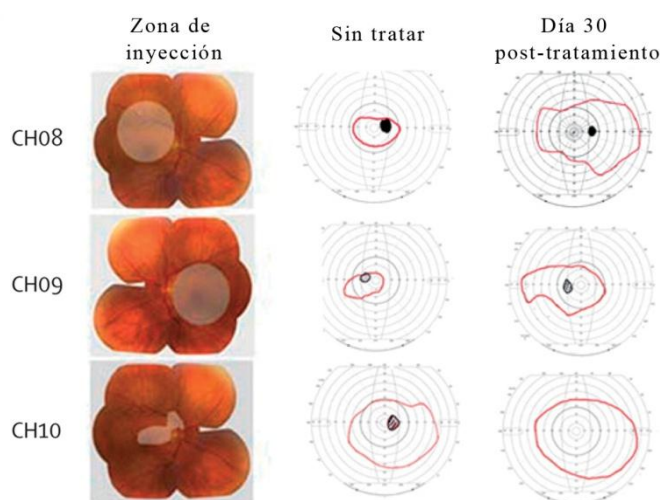
Figura 15. *Ensayo clínico en niños con amaurosis congénita de Leber*. La primera columna muestra la zona de inyección de la retina. La tercera columna muestra la evolución de los campos visuales de Goldman tras el tratamiento con los vectores AAV2-RPE65, respecto a los de la segunda columna (sin tratar).[48]

[47] Cideciyan AV. Leber congenital amaurosis due to *RPE65* mutations and its treatment with gene terapy. Prog Retin Eye Res. 2010;29:398-427.
[48] Maguire AM, High KA, Auricchio A, Wright JF, Pierce EA, Testa F, et al. Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. Lancet. 2009;374:1597-1605.

2.3.3. Coroideremia

La coroideremia es una enfermedad degenerativa de la retina que se caracteriza por una atrofia casi total de la coroides, el epitelio pigmentario retiniano y la retina neurosensorial. Su herencia es recesiva ligada al sexo, afectando a los varones y actuando las mujeres como transmisoras. Comienza en la primera década de vida y progresa muy lentamente. Su frecuencia es de 1:150.000 habitantes y sus manifestaciones clínicas son disminución de la visión nocturna, alteración en fases tardías de la visión central, constricción periférica en los campos visuales y electroretinograma anómalo desde las fases precoces. En la exploración se distingue por la atrofia focal del epitelio pigmentario y coriocapilar que se extiende progresivamente. La mácula suele permanecer intacta hasta fases muy tardías. La mutación del gen CHM que codifica la proteína REP-1 *(Rab Escort Protein 1)* está implicada en la coroideremia.[49,50]

El gen CHM en la coroideremia

La proteína REP-1 (Rab Escort Protein 1) codificada por el gen CHM, está implicada en la activación (mediante prenilación) de la proteína Rab, participando en procesos de endocitosis, distribución intracelular, formación de vesículas, etc. En ausencia de REP-1, Rab no puede realizar su función.
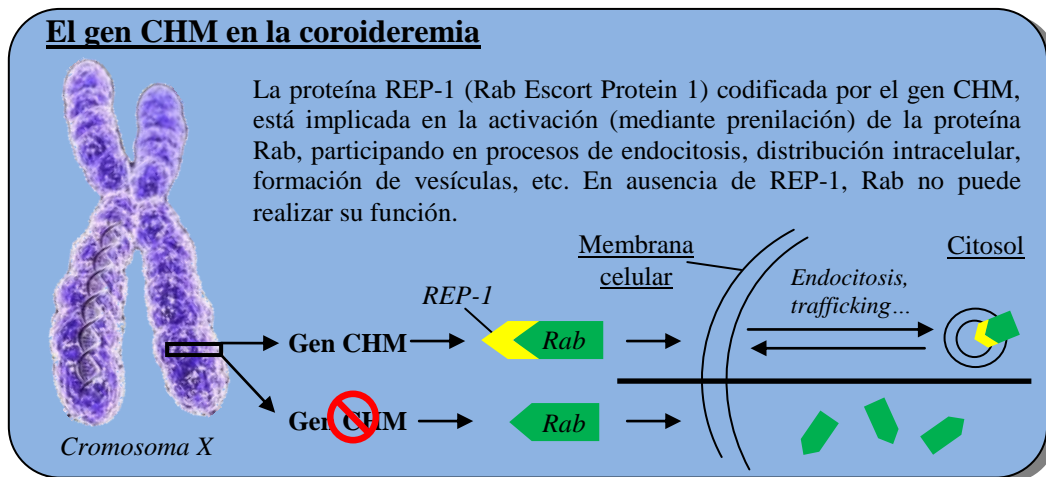
Cuadro 10. *Función del gen CHM en la coroideremia.*

[49] Mura M, Sereda C, Jablonski MM, McDonald IM, Iannaccone A. Clinical and functional findings in choroideremia due to complete deletion of the CHM gene. Arch Ophtamol. 2007;125:1107-1113.

[50] Corbeel L, Freson K. Rab proteins and Rab-associated proteins: major actors in the mechanism of protein-trafficking dissores. Eur J Pediatr. 2008;167:723-729.

Existen varias razones que hacen pensar en la utilidad de la terapia génica en el tratamiento de la coroideremia:

- Se conoce y se ha clonado el gen implicado, identificándose además, al menos 15 mutaciones.
- La coroideremia es una de las pocas retinopatías hereditarias en las que el gen defectuoso puede identificarse mediante examen clínico.
- Los portadores de la enfermedad sufren síntomas mínimos pero pueden ser identificados clínicamente.
- La proteína REP-1 se expresa normalmente en células de otros tejidos además de la retina como, linfocitos y fibroblastos, los cuales pueden ser recogidos y cultivados durante largos periodos de tiempo.

En un estudio en el que se aplicó la terapia génica (Figura 16), se cultivaron "in vitro" linfocitos y fibroblastos de pacientes con esta afección, los cuales no presentaban la proteína REP-1. Se transfectaron las células en cultivo usando como vectores adenovirus con el gen que codifica REP-1 y se constató mediante medidas inmunohistoquímicas la recuperación de los linfocitos y fibroblastos cultivados, que empezaron a expresar REP-1.[51]

Figura 16. *Actividad restaurada de la proteína REP-1.* Se observa el aumento de la actividad de las proteínas RAB en los fibroblastos transfectados con adenovirus (negro) frente a los fibroblastos control (blanco).
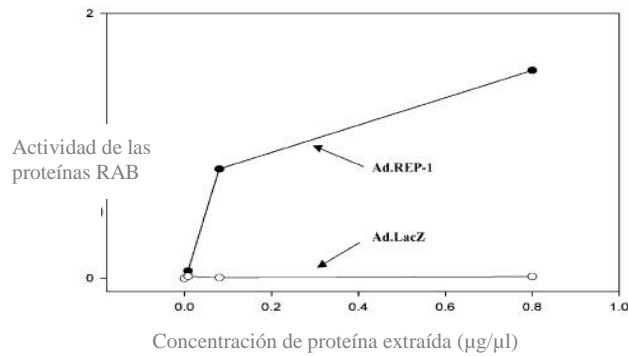
[51] Anand V, Barral DC, Zeng Y, Brunsmann F, Maguire AM, Seabra MC, et al. Gene therapy for choroideremia: in vitro rescue mediated by recombinant adenovirus. Vision Res. 2004;43:919-926.

2.3.4. Retinosquisis Juvenil Ligada al Sexo

La retinosquisis juvenil ligada al cromosoma X es una enfermedad degenerativa de la retina central que tiene un modo de herencia recesivo afectando únicamente a hombres con una prevalencia estimada de 1/5.000 − 1/25.000. Puede desarrollarse a edad muy temprana, pero lo más frecuente es detectarla en pacientes de entre 5 y 10 años por presentar dificultades en la lectura.

Figura 17. *Fondo de ojo con retinosquisis.* En el fondo de ojo con retinosquisis (derecha)*,* se visualiza un desprendimiento de tipo quístico que abarca la parte inferior de la retina y sin afectación macular.
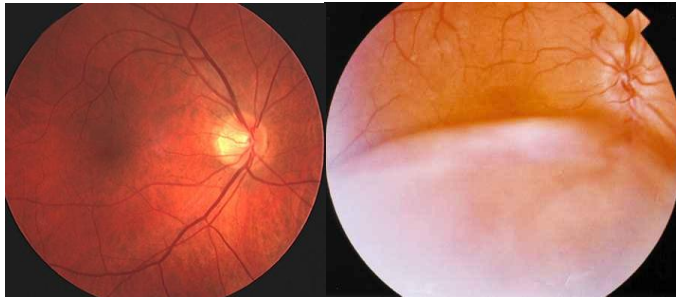
Los principales síntomas que se experimentan desde la infancia son la dificultad para ver en detalle y la disminución de la visión central, que progresa muy lentamente. A veces se produce disminución brusca de la visión debido a hemorragias o desprendimiento de retina. En el centro de la retina se observa una *squisis* (cavidad quística) que puede desarrollarse en la fóvea y en la retina periférica. Los quistes presentan un patrón estrellado y estrías radiales que se han comparado con los radios de una rueda de bicicleta; pueden coalescer para formar una gran cavidad quística central, lo que conduce a una rápida pérdida de visión.

El gen RS1 codifica una proteína de 224 aminoácidos, la retinosquisina. Esta proteína se expresa abundantemente en los fotorreceptores (retina externa), pero la enfermedad típicamente afecta a las capas retinianas más internas que contienen células ganglionares, incluida la capa de fibras nerviosas.
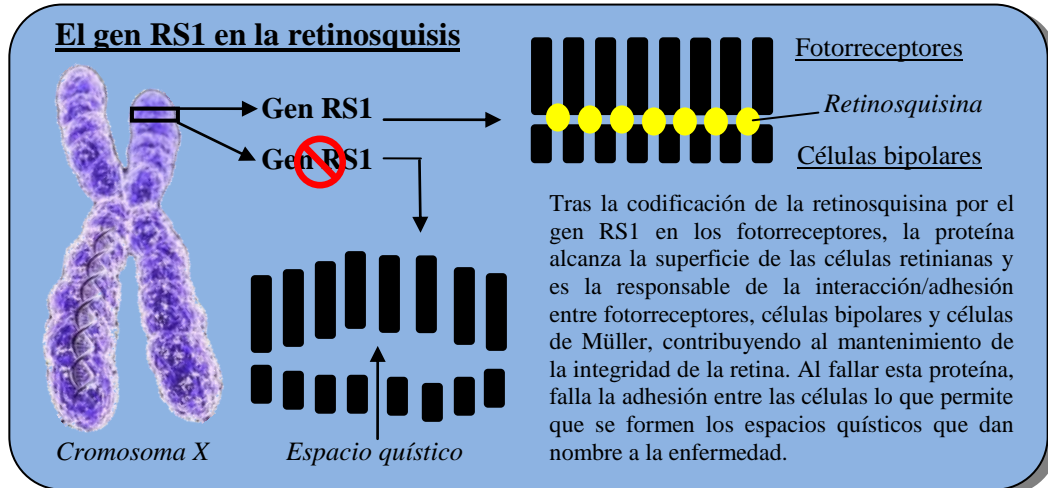
Cuadro 11. *Función del gen RS1 en la retinosquisis.*

Investigadores del Instituto de Genética de la Universidad de Florida han aplicado terapia génica en ratones para tratar la retinosquisis. En este estudio, los investigadores inyectaron una copia buena del gen RS1 humano usando adenovirus asociados (AAV) en el espacio subretiniano del ojo derecho de ratones que tenían el gen defectuoso. El ojo izquierdo de los ratones no se trató. Después de seis meses, los ojos tratados mostraban una mejoría significativa tanto en la función como en la morfología de la retina, con conservación de los fotorreceptores, los cuales sin tratamiento degeneraron progresivamente.[52]
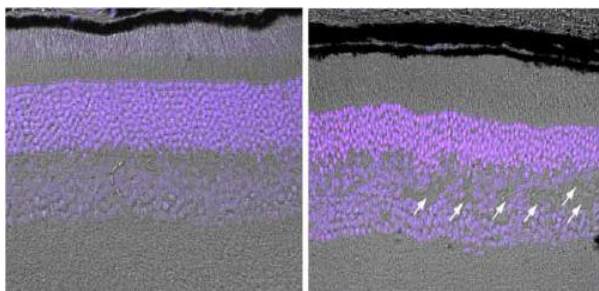
Figura 18. *Terapia génica en la retinosquisis.* Las flechas de la foto de la derecha muestran los numerosos huecos en la capa que forman las células bipolares, lo que evidencia la desestructuración de la retina; huecos que no se observan en la retina tratada con adenovirus asociados portadores del gen RS1 (izquierda). [52]

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[52] Min SH, Molday LL, Seeliger MW, Dinculescu A, Timmers AM, Janssen A, et al. Prolonged Recovery of Retinal Structure/Function after Gene Therapy in an Rs1h-Deficient Mouse Model of X-Linked Juvenile Retinoschisis. Mol Ther. 2005;12:644-651.

2.3.5. Degeneración Macular Asociada a la Edad

Esta enfermedad de la mácula (parte central de la retina) afecta a personas mayores de 50 años disparándose la incidencia a partir de los 70, siendo la causa más común de pérdida de visión por encima de 60 años en los países desarrollados. La visión borrosa central que padecen los afectados por la degeneración macular es el primer síntoma que aparece, siendo más acusado al realizar tareas que necesitan una visión más detallada, como leer. A medida que evoluciona la enfermedad van apareciendo puntos ciegos en la visión central. Si uno de los ojos tiene la enfermedad el otro también la desarrollará. La muerte de los fotorreceptores y de las células del epitelio pigmentario de la retina que se da en esta enfermedad puede estar causada en parte por la formación y el crecimiento de nuevos vasos sanguíneos (angiogénesis) en el ojo y el resultante escape de plasma que forma bolsas de fluido alrededor de la retina (Cuadro 12). La pérdida de visión es aún reversible en este estado de la enfermedad si el proceso de angiogénesis se detiene y se reabsorbe el fluido.[53]
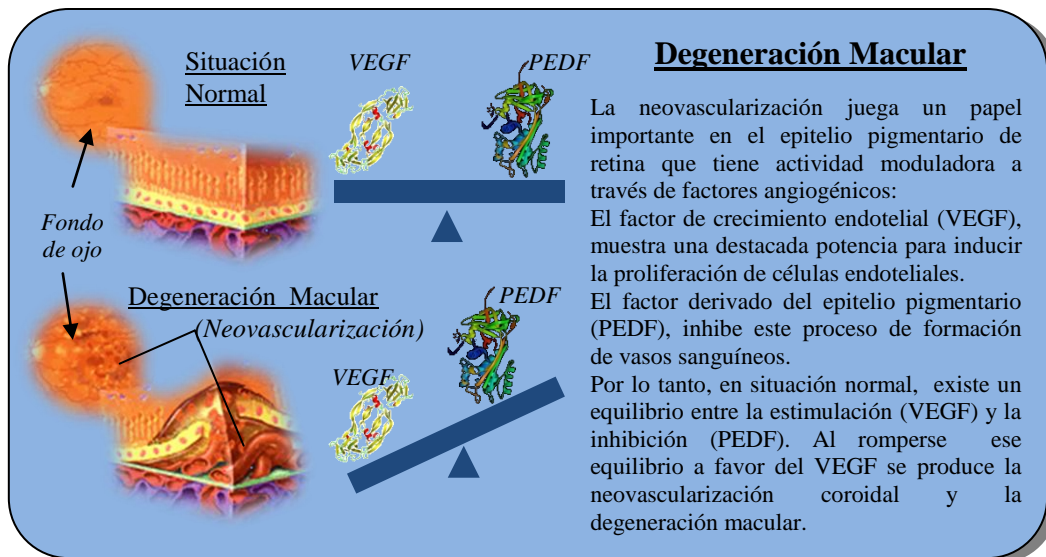
Cuadro 12. *Degeneración macular y neovascularización.*[53]

Se ha realizado un ensayo clínico en el que se inyectaron por vía intravítrea a 28 pacientes con degeneración macular y avanzada neovascularización, vectores adenovirales con el gen SERPINF1 que codifica la proteína PEDF. Los resultados mostraron evidencias de un enlentecimiento en el progreso de la enfermedad; en los pacientes que recibieron la dosis más alta, se detuvo la progresión de las lesiones. Aunque en el 25% de los pacientes se observaron signos inflamatorios leves y transitorios y en 6 pacientes se produjo un incremento de la presión intraocular que se controló mediante terapia tópica, no se detectaron efectos tóxicos graves. Estos resultados ponen de manifiesto una vez más la potencialidad de la terapia génica para el tratamiento de enfermedades oculares como la degeneración macular asociada a la edad.[53,54]

2.3.6. Enfermedad de Stargardt

Usado también el término *fundus flavimaculatus*, la enfermedad de Stargardt comprende un grupo heterogéneo de trastornos hereditarios de la retina, ocasionados por mutaciones en un mismo gen, ABCA4, cuyo modo de herencia es autosómico recesivo. Hasta el 2% de la población tiene mutaciones en este gen, lo que hace que sea la segunda enfermedad hereditaria de la retina más frecuente tras la retinosis pigmentaria.

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[53] Ohno-Matsui K, Morita I, Tombran-Tink J, Mrazek D, Onodera M, Uetama T, et al. Novel mechanism for age-related macular degeneration: an equilibrium shift between the angiogenesis factors VEGF and PEDF. J Cell Physiol. 2001;189:323-333.

[54] Campochiaro PA, Nguyen QD, Shah SM, Klein ML, Holz E, Frank RN, et al. Adenoviral vector-delivered pigment epithelium-derived factor for neovascular age-related macular degeneration: results of a phase I clinical trial. Hum Gene Ther. 2006;17:177-179.

Inicialmente los pacientes se quejan de una disminución de la visión, la cual es borrosa en su parte central. La pérdida de visión periférica no es destacable. En esta etapa, la retina puede mostrar mínimos cambios atróficos en el epitelio pigmentario y al progresar la enfermedad se desarrolla un fondo oscuro o de color bronce causado por el excesivo depósito de lipofucsina en el epitelio, que además impide la visualización de los detalles coroideos (silencio coroideo). La acumulación de este pigmento también provoca unas características manchas amarillentas ("flecks") que representan alargamientos de las células del epitelio pigmentario debido al acúmulo de la lipofucsina.
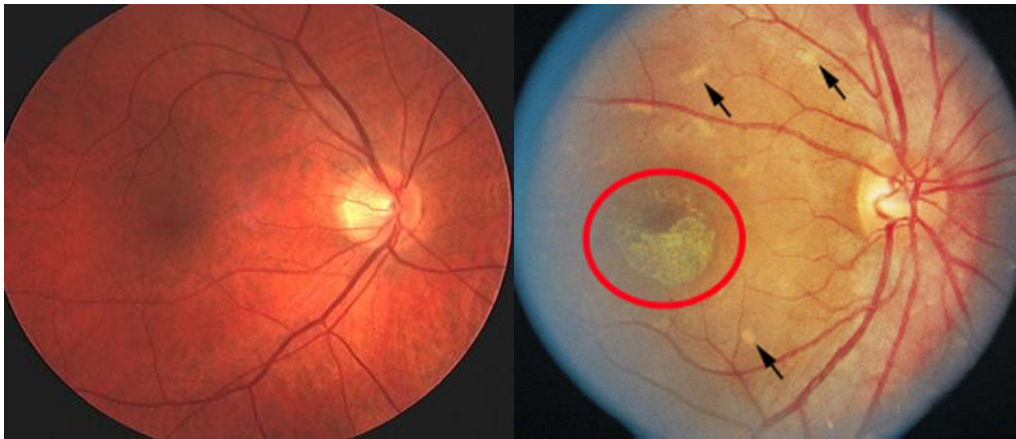
Figura 19. *Fondo de ojo normal (izquierda) y fondo de ojo con Enfermedad de Stargardt (derecha).* Se pueden apreciar los signos clásicos de esta enfermedad: mácula con apariencia bronceada (círculo rojo), "flecks" de tono amarillento (flechas) y ausencia de los detalles coroideos en el polo posterior (silencio coroideo).

Variables en forma, tamaño y distribución, los "flecks" son ovales en forma de cúpula; su color cambia de amarillo a gris y pueden aparecer más grandes y menos definidos. A medida que progresa la enfermedad, el continuado acúmulo de lipofucsina destruye las células del epitelio pigmentario, el cual resulta atrofiado. La Figura 19 muestra los cambios que presenta un fondo de ojo afectado por la enfermedad de Stargardt respecto a uno normal.

El depósito de lipofucsina en el epitelio pigmentario de retina está causado por mutaciones en el gen ABCA4, que codifica la Rim Protein, cuyo funcionamiento se explica en el Cuadro 13.[55,56,57,58]
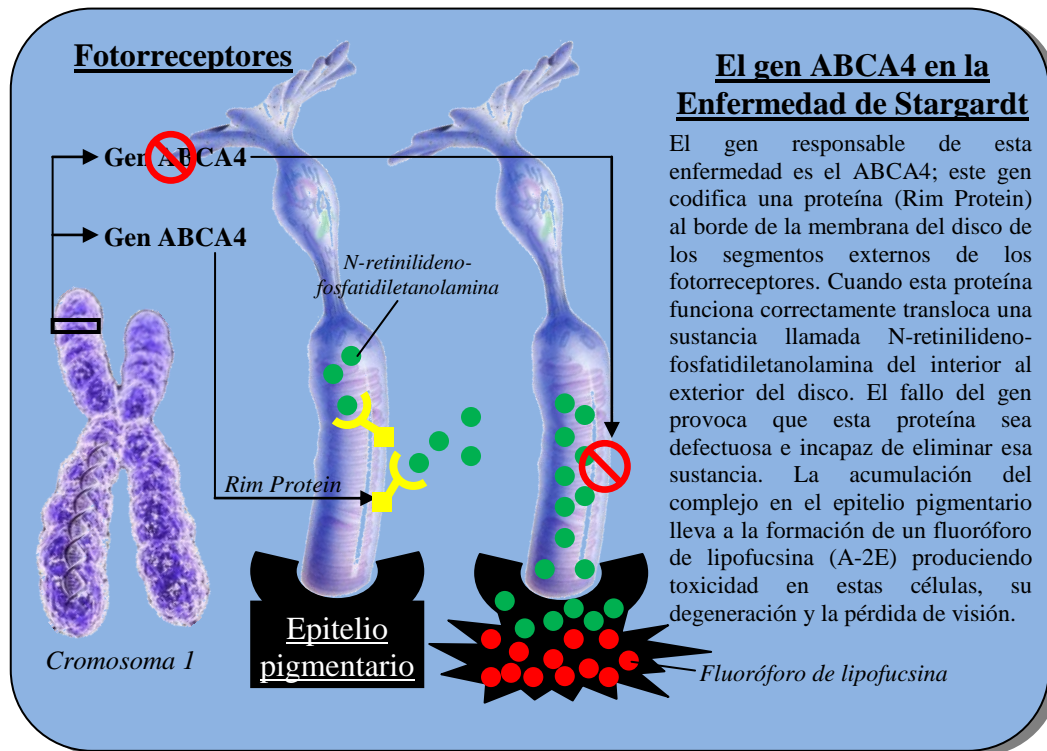
Cuadro 13. *Función del gen ABCA4 en la enfermedad de Stargardt.*

[55] Weng J, Mata NL, Azarian SM, Tzekov RT, Birch DG, Travis GH. Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knock-out mice. Cell. 1999;98:13-23.

[56] Michaelides M, Hardcastle AJ, Hunt DM, Moore AT. Progressive cone and cone-rod dystrophies: phenotypes and underlying molecular genetic basis. Survey Ophthalmol. 2006;51:232-258.

[57] Kaminski WE, Piehler A, Wenzel JJ. ABCA-subfamily transporters: structure, function and disease. Biochim Biophys Acta. 2006;1762:510-524.

[58] Sparrow JR, Fishkin N, Zhou J, Cai C, Jang YP, Krane S, et al. A2E, a byproduct of the visual cycle. Vision Res. 2003;43:2983-2990.

Los pacientes con enfermedad de Stargardt se pueden dividir en:

- *Fondo ocular de bronce y silencio coroideo.* Es el estado más precoz. El excesivo pigmento en el epitelio pigmentario produce el color bronce en el fondo del ojo y oscurece los detalles de fondo de la coroides.

- *Maculopatía atrófica con o sin "flecks" amarillentos.* Las lesiones amarillas y el almacenamiento de lipofucsina es evidente. En el epitelio pigmentario se puede desarrollar hipertrofia, hiperplasia, metaplasia fibrosa y atrofia. Es posible que haya alargamiento en la adaptación a la oscuridad, fotofobia y distrofia en los conos.

- *Maculopatía atrófica con signos y síntomas tardíos de retinosis pigmentaria.* Similar al grupo anterior pero en edades más avanzadas. Hay signos y síntomas de retinosis pigmentaria: nictalopia, pérdida del epitelio pigmentario, estrechamiento de los vasos retinianos y distrofia en fotorreceptores por mutaciones en el gen ABCA4.

- *"Flecks" amarillentos no asociados a atrofia macular.* Lesiones amarillentas centrales y paracentrales que presentan mínima evidencia de atrofia en el epitelio pigmentario de la retina. Suele haber silencio coroideo.[59]

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[59] Kapadia ODS. Stargardt's macular dystrophy. Clinical Eye and Vision Care. 2000;12:71-78.

2.3.7. Retinosis pigmentaria

La retinosis pigmentaria es un término que engloba a un grupo de enfermedades hereditarias que se caracterizan por la degeneración progresiva que sufren las células fotorreceptoras de la retina. Esta enfermedad, que aproximadamente tiene una incidencia en la población de 1/3.500, es muy heterogénea y aún no existe un tratamiento que pueda prevenirla, estabilizarla o revertirla.

El proceso degenerativo afecta inicialmente a los bastones y a las células del epitelio pigmentario de la retina. Los primeros síntomas que sufren los pacientes afectados por la retinosis pigmentaria son la ceguera nocturna (se adaptan mal a la oscuridad) y pérdida de la visión periférica (se produce una visión en túnel). En las fases finales de la enfermedad los conos también resultan afectados (pérdida de la visión central), causando la ceguera en un grupo importante de pacientes.
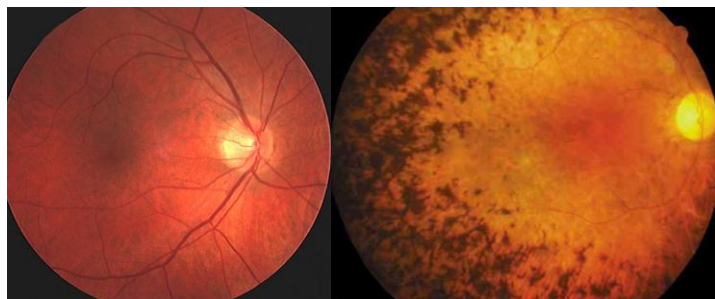
Figura 20. *Fondo de ojo con retinosis pigmentaria.* Al comparar el fondo normal (izquierda) con el afectado con retinosis (derecha), se observan las manifestaciones típicas de la enfermedad: atenuación del árbol vascular, palidez de la pupila y pigmentaciones en la periferia.

La retinosis pigmentaria presenta varios modelos de herencia:

- *Modelo autosómico dominante (ADRP):* representa el 15%-20% de los casos. Aparecen uno o más afectados en herencias sucesivas, afectando tanto a hombres como a mujeres.

- *Modelo autosómico recesivo (ARRP):* el 20%-25% de casos. Los progenitores no padecen la enfermedad pero sí trasmiten el gen anormal, que al coincidir en el hijo desarrolla la enfermedad. En estos casos la consanguinidad suele ser frecuente.

- *Modelo ligado al cromosoma X (XLPR):* el 10%-15% de casos de retinosis pigmentaria. Las mujeres, generalmente asintomáticas, son las portadoras y trasmiten la enfermedad a los hijos varones que son los que la padecen.

- *Casos esporádicos:* Son el 44%-55% del total y no pueden clasificarse genéticamente.

Las mutaciones más comunes se dan en los genes que desempeñan su función en la *fototransducción*. Destacan el gen RHO que codifica la proteína rodopsina (Cuadro 14), y los genes PDE6A y PDE6B implicados en la GMPc fosfodiesterasa. Otros genes responsables de la retinosis pigmentaria son el RP1 (factores de transcripción), RPGR (proteínas transportadoras), etc.
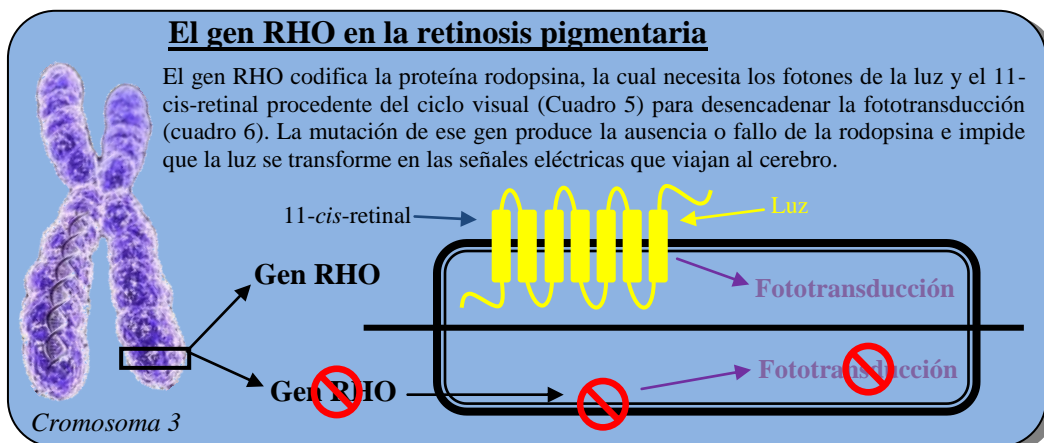
Cuadro 14. *Función del gen RHO en la retinosis pigmentaria.*

La genética de la retinosis pigmentaria es muy heterogénea y sólo se ha conseguido identificar una parte de la gran cantidad de genes que están implicados en esta enfermedad; aún así, en un 60% de casos de retinosis pigmentaria se desconoce la posible causa genética.

Los genes implicados (Tabla 2) se encuentran en las células del epitelio pigmentario y en los fotorreceptores, y pueden ser agrupados según la función que lleven a cabo. [60,61,62,63]

Gen	Función		Gen	Función
CRX	Transcripción		LRAT	Metabolismo de retinoides
FSCN2	Morfología		MERTK	Recambio de discos
IMPDHI	Crecimiento celular		NR2E3	Transcripción
NRL	Transcripción		PDE6A	Fototransducción
PDC	Fototransducción		PDE6B	Fototransducción
PRPF31	Corte del pre-mRNA		RGR	Metabolismo de retinoides
RDS	Estructura		RLBP1	Metabolismo de retinoides
RHO	Fototransducción		RPE65	Metabolismo de retinoides
ROM1	Estructura		SAG	Fototransducción
RP1	Transcripción		TULP1	Transcripción
RP17	Corte del pre-mRNA		USH2A	Desarrollo de retina
ABCA4	Catabolismo		CERKL	Metabolismo
CNGA1	Fototransducción		RP2	Proteínas
CNGB1	Fototransducción		RPGR	Proteínas transportadoras
CRB1	Transcripción			

Tabla 2. *Genes implicados en la retinosis pigmentaria y función en la que participan.*

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El Cuadro 15 recoge un resumen de las principales enfermedades degenerativas de la retina mostrándose como es la visión en los enfermos que padecen estas retinopatías.

Cuadro 15. *Visión en las distintas retinopatías; al final, la mayoría conducen a la ceguera.*

OBJECTIVES
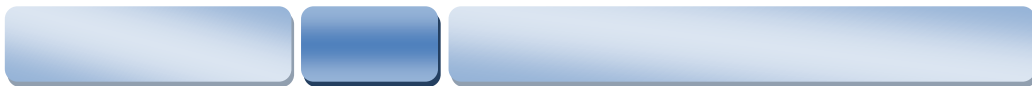
Gene therapy is a rapidly advancing field with great potential for the treatment of genetic and acquired systemic diseases. This therapy involves two systems for the targeted delivery of therapeutic genetic material into cells: viral and non-viral vectors. The former are the most effective but present many safety-related problems such as immunogenicity and oncogenicity. The latter, in contrast, are much safer, cheaper and more reproducible than viral vectors. Furthermore, non-viral vectors have no limitations in the size of DNA they can transport. However, the efficacy of these vectors is still low; thus, the key challenge of gene therapy is to develop non-viral vectors in order to improve their effectiveness.

OBJECTIVES

The main objective of this work is to develop non-viral vectors based on solid lipid nanoparticles. It will be carried out following these steps:

1. To design and optimize non viral vectors taking into consideration internalization processes and intracellular disposition of genetic material incorporated in nanoparticulate systems.

2. To test "in vitro" the utility of vectors loading therapeutic genes.

3. To evaluate the local transfection capacity of vectors "in vivo" after ocular application in rats by different administration routes.

4. To evaluate the systemic transfection capacity of vectors "in vivo" after intravenous administration in mice.

La terapia génica se perfila como una prometedora herramienta para el tratamiento tanto de enfermedades genéticas como adquiridas. Esta terapia comprende dos sistemas para la administración de material genético: vectores virales y vectores no virales. Los primeros son los más eficaces pero presentan problemas de seguridad como la inmunogenicidad o la oncogenicidad. Los segundos, en cambio, son mucho más seguros, baratos y reproducibles; además los vectores no virales no tienen limitación en el tamaño del ADN a transportar. Sin embargo, su eficacia es aún baja; por lo tanto, el principal reto de la terapia génica es desarrollar vectores no virales para mejorar su efectividad.

OBJETIVOS

El principal objetivo de este trabajo es desarrollar vectores no virales basados en nanopartículas sólidas lipídicas. Será llevado a cabo siguiendo las siguientes etapas:

1. Diseñar y optimizar vectores no virales teniendo en cuenta los procesos de internalización y la disposición intracelular del material genético incorporado en los sistemas nanoparticulares.

2. Probar "in vitro" la utilidad de los vectores cargados con genes terapéuticos.

3. Evaluar "in vivo" la capacidad de transfección local de los vectores tras su aplicación ocular en ratas por diferentes vías de administración

4. Evaluar "in vivo" la capacidad de transfección sistémica de los vectores tras su administración intravenosa en ratones.
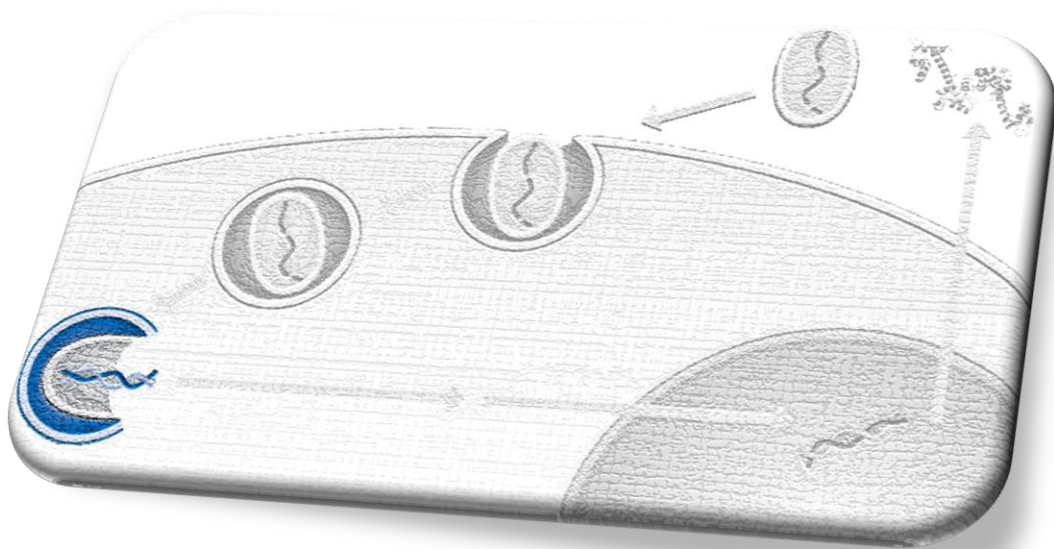
UNDERSTANDING THE MECHANISM OF PROTAMINE IN SOLID LIPID NANOPARTICLE-BASED LIPOFECTION: THE IMPORTANCE OF THE ENTRY PATHWAY

Authors

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ABSTRACT
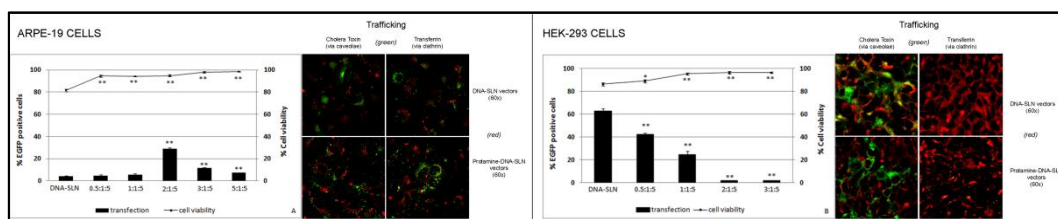
The aim of our study was to evaluate the effect of protamine on the transfection capacity of SLN (solid lipid nanoparticles) by correlating it to the internalization mechanisms and intracellular trafficking of the vectors.

Vectors were prepared with SLN, DNA and protamine. ARPE-19 and HEK-293 cells were used for the evaluation of the formulations.

Protamine induced a 6-fold increase in the transfection of SLNs in retinal cells due to the presence of nuclear localization signals (NLS), its protection capacity and a shift in the internalization mechanism from caveolae/raft-mediated to clathrin-mediated endocytosis. However, protamine produced an almost complete inhibition of transfection in HEK-293 cells.

In spite of the high DNA condensation capacity of protamine and its content in NLS, this does not always lead to an improvement in cell transfection since it may impair some of the limiting steps of the transfection processes.

Keywords: Solid lipid nanoparticles; Transfection; Intracellular trafficking; Protamine; Endocytosis;

1. INTRODUCTION

Gene therapy, which involves systems for the targeted delivery of DNA into cells, has a great potential for treating a large number of diseases.[1,2,3,4] Non-viral vectors are safer, cheaper, and more reproducible than viral vectors, and have no limitation in the size of DNA they can transport, but their transfection efficacy is still low.[5] Therefore, the enhancement of non-viral vectors is a priority in the field of gene therapy, in order to manage effective and safe vectors.

Non-viral vectors based on cationic lipids are being extensively studied,[6,7,8,9] and, among lipidic systems, solid lipid nanoparticles (SLN) have become a promising strategy as gene delivery systems. In previous studies, we showed the capacity of SLN for transfection "in vitro" [5,10] and "in vivo", [11] and from the point of view of

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[10] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles for retinal gene therapy: Transfection and intracellular trafficking in RPE cells. Int J Pharm. 2008;360:177-183.

application, SLN have good stability and are subject to be lyophilized,[12] which facilities the industrial production.

Transfection efficacy is conditioned by the entry and posterior intracellular trafficking of gene delivery vectors. These processes are cell line dependent, but there are steps common to all cells:[13] binding to cell surface by electrostatic interactions between the positively charged systems and the negative charges of the cell membrane, entry into the cell by following a particular pathway,[14,15] release of DNA into the cytoplasm and crossing the nuclear envelope to reach the cellular machinery for protein synthesis. The entry into the nucleus is, in general, quite difficult, as the nuclear membrane is a selective barrier to molecules longer than 40KDa, and plasmids surpass that size. There are two mechanisms those molecules can use to overcome that barrier: the disruption of the nuclear membrane during mitosis, which is conditioned by the division rate of the targeted cells, or the import through the nuclear pore complex (NPC). This latter mechanism requires nuclear localization signals (NLS), which can be used to improve transfection by non-viral vectors.[16]

NLS peptides have sequences rich in basic aminoacids (lysine or arginine) that promote active nuclear transport through the NPC, mediated by importin α –

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importin β interactions.[17] Peptides with arginine residues in their sequences have shown translocation activity and, those containing 6 or 8 consecutive arginine residues, exhibited the maximum internalization and accumulation in the nucleus.[18,19] Among these peptides, protamine sulphate is an USP (United States Pharmacopeia) compound isolated from the sperm of mature fish. It condenses DNA and presents sequences of 6 consecutive arginine residues,[20] which make this peptide able to translocate molecules such as DNA from the cytoplasm to the nucleus of living cells. This feature would solve the passage of DNA from the cytoplasm into the nucleus. Moreover, protamine improves intra-nuclear transcription.[21]

The present study considers the effect of protamine on the transfection capacity of SLN composed by the core lipid Precirol® ATO 5, and the surfactants DOTAP and Tween 80 as surface components. Protamine-DNA-SLN complex was characterized in terms of size, superficial charge and DNA protection capacity. The mechanism of internalization and the intracellular trafficking of the vectors were also studied into cell lines with different division rates and related to the transfection capacity of the vectors.

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2. MATERIALS AND METHODS

2.1. Materials

Precirol® ATO 5 was provided by Gattefossé (Madrid, Spain). 1,2-Dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) was acquired from Avanti Polar Lipids, Inc. Deoxyribonuclease I (DNase I), lauryl sulphate sodium (SDS), Nile Red and protamine sulphate were purchased from Sigma-Aldrich (Madrid, Spain). Tween 80 was provided by Vencaser (Bilbao, Spain), and dichloromethane, by Panreac (Barcelona, Spain).

The plasmid pCMS-EGFP, which encodes the enhanced green fluorescent protein (EGFP), was purchased from BD Biosciences Clontech (Palo Alto, California, US) and amplified by Dro Biosystems S.L. (San Sebastian, Spain). The labeling of the plasmid pCMS-EGFP with ethidium monoazide (EMA) was carried out by Dro Byosystems S.L. (San Sebastian, Spain).

The gel electrophoresis materials were acquired from Bio-Rad (Madrid, Spain).

Cell culture reagents were purchased from LGC Promochem (Barcelona, Spain). Antibiotic Normocin™ was acquired from InvivoGen (San Diego, California, US). The BD Viaprobe kit was provided by BD Biosciences (Belgium). Hoechst 33258, AlexaFluor488-Cholera toxin and AlexaFluor488-Transferrin were provided by Molecular Probes (Barcelona, Spain), and Fluoromount-G by SouthernBiotech (Coultek, España).

2.2. Production of solid lipid nanoparticles (SLNs)

The SLN were produced by a solvent emulsification-evaporation technique previously described by del Pozo-Rodríguez et al. 2007.[22]

Briefly, Precirol® ATO 5 was dissolved in dichloromethane (5% w/v), and then emulsified by sonication (Branson Sonifier 250, Danbury) for 30 seconds at 50 W in an aqueous phase containing DOTAP (0.4% w/v) and Tween 80 (0.1% w/v). The organic solvent was removed by evaporation and SLN suspension was formed upon solidification of the Precirol® ATO 5. The SLN were washed by centrifugation (3,000 rpm, 20 minutes, x 3) using Millipore (Madrid, Spain) Amicon® Ultra centrifugal filters (100,000 MWCO).

2.3. Binding of protamine to DNA

pCMS-EGFP plasmid was added to an aqueous solution of protamine at protamine to DNA ratios (w/w) of 0.25:1, 0.5:1, 1:1, 2:1 and 5:1.

The resulting complexes were diluted in Milli-Q™ water up to a final concentration of 0.03 μg DNA/μL, and then subjected to electrophoresis on a 1% agarose gel (containing ethidium bromide for visualization) for 30 min at 120 V. The bands were observed with an Uvidoc D-55-LCD-20M Auto transilluminator (Uvitec).

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2.4. Preparation of vectors

DNA-SLN vector was obtained by mixing the pCMS-EGFP plasmid with an aqueous suspension of SLN. The SLN to DNA ratio, expressed as the ratio of DNA to DOTAP (w/w), was fixed at 1:5.

Protamine-DNA-SLN vector was prepared by first binding protamine to DNA at the ratios (w:w:w) 0.5:1:5, 1:1:5, 2:1:5, 3:1:5 and 5:1:5 under agitation for 30 minutes. Then protamine-DNA complexes were put in contact for 15 minutes with a suspension of previously prepared SLN, and electrostatic interactions between complexes and SLN led to the formation of the vector. This vector has protamine-DNA complexes adsorbed on the nanoparticle surface.

2.5. Size and zeta potential measurements

Sizes of SLN, DNA-SLN (1:5) and protamine-DNA-SLN (2:1:5) vectors were determined by photon correlation spectroscopy (PCS). Zeta potentials were measured by laser Doppler velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). All samples were diluted in 0.1 mM NaCl (aq.).

2.6. DNase I protection and SDS-induced release of DNA

DNase I was added to DNA-SLN and protamine-DNA-SLN vectors to a final concentration of 1 U DNase I/2.5 μg DNA. The mixtures were then incubated at 37 ºC for 30 min. Afterwards, 1% SDS solution was added to release the DNA from the SLN. Additionally, the vectors were treated only with SDS to study the ability of

DNA to be released from the vectors. The samples were then analyzed by electrophoresis on agarose gel (described above), and the integrity of the DNA in each sample was compared to a control of untreated DNA.

2.7. Cell culture and transfection protocol

"In vitro" assays were performed with two different cell lines: a human embryonic kidney (HEK-293) cell line and a human retinal pigmented epithelial (ARPE-19) cell line, obtained from the American Type Culture Collection (ATCC).

HEK-293 cells were maintained in Eagle's Minimal Essential medium with Earle's BSS and 2 mM L-glutamine (EMEM) supplemented with 10% heat-inactivated horse serum and 1% Normocin™. Cells were incubated at 37ºC with 5% CO_2 in air and subcultured every 2-3 days using trypsin/EDTA. For transfection HEK-293 cells were seeded on 24 well plates at density of 150,000 per well and allowed to adhere overnight.

ARPE-19 cells were maintained in Dulbecco's Modified Eagle's Medium-Han's Nutrient Mixture F-12 (1:1) medium (D-MEM/F-12) supplemented with 10% heat-inactivated fetal calf serum and 1% Normocin™ antibiotic solution. Cells were incubated at 37 ºC under 5% CO_2 atmosphere and subcultured every 2-3 days using trypsin-EDTA. For transfection, ARPE-19 cells were seeded on 12 well plates at a density of 30,000 cells per well and allowed to adhere overnight.

The formulations were diluted in HBS and added to the cell cultures. In all cases, 2.5 μg of DNA were added. The cells were incubated with the vectors at 37 ºC, and after 4 h, the medium containing the complexes in the wells was refreshed with 1

mL of complete medium. The cells were then allowed to grow for another 72 h. Transfection efficacy was quantified at 12 h, 24 h, 48 h, 72 h and 1 week.

As control, DNA-SLN vector without protamine whose transfection ability was evaluated in previous works was also assayed.[10,22]

2.8. Flow cytometry mediated analysis of transfection efficacy and cell viability

At the end of the incubation period, the cells were washed once with 400 μL of PBS, and then detached with 400 μL of 0.05% trypsin-EDTA. The cells were then centrifuged at 1,500 x g, and then resuspended with PBS and directly introduced into a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, California, US). For each sample, 10,000 events were collected.

Transfection efficacy was quantified by measuring the fluorescence of EGFP at 525 nm (FL1). For cell viability measurements, the BD Via-Probe kit was employed. The reagent (5 μL) was added to each sample, and after 10 minutes of incubation, the fluorescence corresponding to dead cells was measured at 650 nm (FL3).

2.9. Cellular uptake of DNA-SLN and protamine-DNA-SLN vectors

Entry of vectors into the cells was studied quantitatively by flow cytometry. For this purpose pCMS-EGFP was labeled with ethidium monoazide (DNA-EMA), as previously described.[10]

24 hours after the addition of vectors, cells were washed three times with PBS and detached from plates. Cells incorporating either DNA-EMA-SLN or protamine-DNA-EMA-SLN vectors were quantified by flow cytometry at 650 nm (FL3). For each sample, 10,000 events were collected.

2.10. Internalization mechanism

First, endocytosis mechanisms were evaluated in both cell lines by flow cytometry and the entry of AlexaFluor488-Cholera Toxin and AlexaFluor488-Transferrin, which are markers of caveolae raft mediated endocytosis and clathrin mediated endocytosis, respectively, was quantified (FL1).

The endocytic processes involved in the internalization of the non-viral systems were analyzed qualitatively by colocalization studies with both markers. SLN was labeled with the fluorescent dye Nile Red ($\lambda = 590$ nm) according to a previously reported method.[23] Briefly, Nile Red was incorporated into the dichloromethane employed to prepare SLN by the emulsification–evaporation technique described above. Cells were seeded in coverslips and co-incubated for 2 hours with Nile Red labeled vectors and either AlexaFluor488-Cholera toxin (10 μg/ml) or AlexaFluor488-Transferrin (50 μg/ml). Next, medium was removed and cells were washed with PBS and fixed with paraformaldehyde 4%, which did not interact with the fluorescence of Nile Red or AlexaFluor488. Preparations were mounted on Fluoromount G, and after air-drying, images were obtained with an Olympus

[23] Borgia SL, Regehly M, Sivaramakrishan R, Mehnert W, Korting HC, Danker K, et al. Lipid nanoparticles for skin penetration enhancement-correlation to drug localization within the particle matrix as determined by fluorescence and parelectric spectroscopy. J Control Release. 2005;110:151-163.

Fluoview FV500 confocal microscope using sequential acquisition to avoid overlapping of fluorescent emission spectra. Confocal laser scanning microscopy study (CLSM) images were captured in the General Service of Analytical Microscopy and High Resolution in Biomedicine of the University of the Basque Country (UPV-EHU). Colocalization results were estimated by means of Manders Overlap Coefficient (R), where $0.6 \leq R \leq 1.0$ indicates colocalization (overlap of the signals).[24] The value of every coefficient was obtained from 15 images.

2.11. Detection of intracellular EMA-labeled DNA by fluorescence microscopy

In order to follow the DNA into the cytoplasm, cells were seeded in culture plates and treated with vectors containing DNA-EMA. Prior to the observation of the samples through the microscope, nucleuses were labeled with Hoechst 33258. Images were analyzed with an inverted fluorescent microscopy (model EclipseTE2000-S, Nikon).

2.12. Statistical analysis

Statistical analysis was performed with SPSS 17.0 (SPSS®, Chicago, IL, USA). Normal distribution of samples was assessed by the Shapiro–Wilk test and homogeneity of variance by the Levene test. The different formulations were compared with ANOVA and the student´s t test, whereby differences were considered statistically significant at $p < 0.05$.

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3. RESULTS

3.1. Binding of protamine to DNA

The gel in Figure 1A illustrates that the capacity of protamine to condensate DNA depends on the protamine to DNA ratio. In lanes 1–3 (protamine to DNA ratios from 0.25:1 to 1:1), the intensity of the bands indicates that most DNA was free. However, in lines 4 and 5 (protamine to DNA ratios 2:1 and 5:1, respectively) no band is present in the gel, indicating that all DNA was completely bound to protamine.

3.2. Characterization

3.2.1. Particle size and zeta potential of the formulations

When protamine-DNA (ratio 2:1) complexes were bound to SLN, protamine did not induce any significant change in surface charge or particle size of SLN, being the measures in the region of +35 mV (S.D. =2.40) and 250 nm (S.D. =43.20), with normal distribution and polydispersion index lower than 0.4. The labeling of vectors with Nile Red or EMA did not induce changes in the particle size or zeta potential (p>0.05).

3.2.2. DNase I protection and SDS-induced release of DNA

DNA formulated in DNA-SLN or protamine-DNA-SLN vectors was protected from nuclease action (gel electrophoresis in figure 1B). The addition of protamine to the formulations increased the DNA protection capacity of the DNA-

SLN formulation. Figure 1C shows that after treatment with SDS, DNA release is more difficult as protamine to DNA ratio increases.
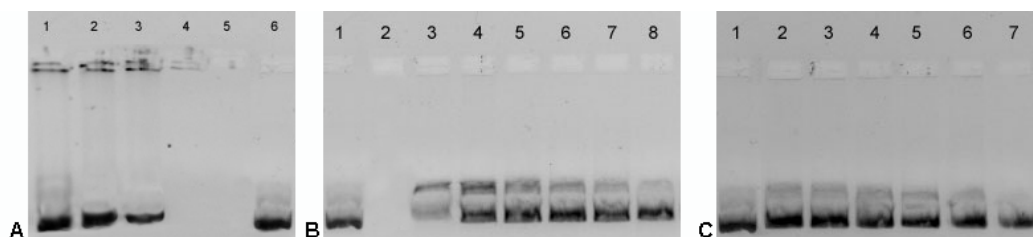
Figure 1.

Figure 1A. Binding efficiency of DNA with protamine at different protamine-DNA ratios (w/w) studied by agarose gel electrophoresis. Lane 1= 0.25:1; lane 2= 0.5:1; lane 3= 1:1; lane 4= 2:1; lane 5= 5:1; lane 6= free DNA.

Figure 1B. Protection of DNA by DNA-SLN and protamine-DNA-SLN vectors from DNase digestion at different protamine-DNA-SLN ratios (w/w/w) visualized by agarose gel electrophoresis. Vectors were treated with DNase I. Lane 1= non-treated free DNA; Lane 2= DNase-treated free DNA; Lane 3= DNA-SLN vector; Lane 4= 0.5:1:5; Lane 5= 1:1:5; Lane 6= 2:1:5; Lane 7= 3:1:5; Lane 8= 5:1:5.

Figure 1C. Release of DNA by DNA-SLN and protamine-DNA-SLN at different protamine-DNA-SLN ratios (w/w/w) visualized by agarose gel electrophoresis. Vectors were treated with SDS. Lane 1= free DNA; Lane 2= DNA-SLN vector; Lane 3= 0.5:1:5; Lane 4= 1:1:5; Lane 5= 2:1:5; Lane 6= 3:1:5; Lane 7= 5:1:5.

3.3. Transfection and cell viability studies in ARPE-19 cells and HEK-293 cells

Prior to the evaluation of the efficacy of protamine-containing vectors, we showed that protamine-DNA complexes were not able to transfect cells. As observed in Figure 2A (bars), transfection levels in ARPE-19 cells depend on the protamine content, reaching the highest level (29% EGFP positive cells) with the protamine-DNA-SLN formulation prepared at 2:1:5 ratio ($p < 0.01$). However, the vector without

protamine (DNA-SLN) produced only 5% transfected cells. Cell viability (lines in Figure 2A) was maintained around 95% with all the formulations bearing protamine, with a significantly higher viability ($p<0.01$) than that in cells treated with the DNA-SLN vector (80% viability).
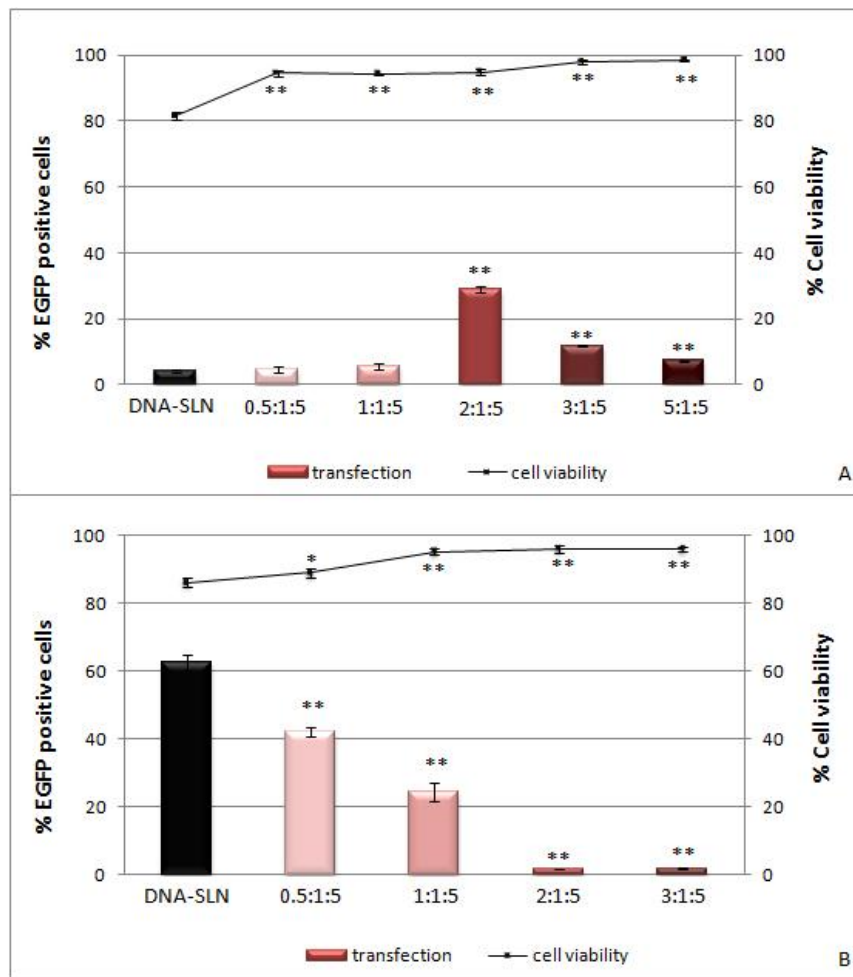
Figure 2. Transfection (bars) and cell viability (line) for each formulation assayed in ARPE-19 cells (A) and HEK-293 cells (B) 72 hours after the addition of vectors; the SLN to DNA ratio (w/w) was 5:1 in all cases, and the protamine to DNA ratio varied from 0.5:1 to 5:1. Error bars represent S.D. (n = 3).
*$p<0.05$ respect to DNA-SLN formulation.
**$p<0.01$ respect to DNA-SLN formulation.

Contrary to ARPE-19 cells, the highest transfection level in HEK-293 cells was achieved with the formulation prepared without protamine (Figure 2B). The incorporation of this peptide to the vectors caused a significant decrease ($p<0.01$) in the transfection levels, although cell viability (line in figure 2B) was higher with the formulations containing protamine ($p<0.01$).

Figure 3 shows transfection of vectors over time in ARPE-19 cells. Transfection levels were significantly higher ($p<0.01$) with the formulation containing protamine. Seven days after the addition of vectors, transfection did not increase in HEK-293 cells with any of the formulations (data not shown).
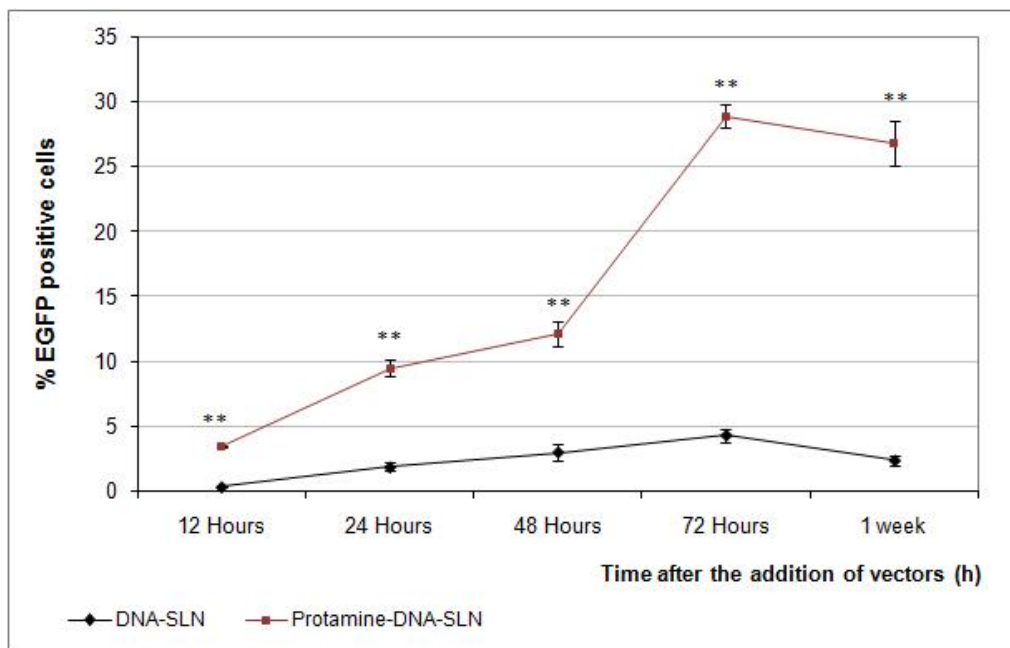
Figure 3. Transfection of non-viral vectors in ARPE-19 cells over time. The SLN to DNA ratio (w/w) was 5:1 and the protamine to DNA ratio 2:1. Error bars represent S.D. (n = 3). ** $p<0.01$ respect to DNA-SLN formulation.

3.4. Cellular uptake of non-viral vectors

The presence of protamine at ratio protamine:DNA 2:1 in the vector decreased the entry into HEK-293 cells from 75% to 45% DNA-EMA positive cells; however, protamine did not modify the cellular uptake of vectors in ARPE-19 cells (77% vs. 72% DNA-EMA positive cells, with DNA-SLN 1:5 and protamine-DNA-SLN 2:1:5, respectively).

3.5. Internalization mechanism

Figure 4 features the flow cytometry results and confocal images of ARPE-19 cell after the treatment with the endocytosis markers and with the vectors (DNA-SLN and protamine-DNA-SLN at a ratio of 2:1:5).

Colocalization with both transferrin and cholera toxin was observed with the two vectors (with and without protamine), indicating that both clathrin and caveolae were involved in the internalization process. However, transferrin colocalization obtained when the cells are treated with the formulation containing protamine (R= 0.641 ± 0.039) is higher than colocalization achieved by the formulation without protamine (R= 0.424 ± 0.124).

Figure 5 features the entry of endocytosis markers and the colocalization study in HEK-293 cells. The cholera toxin showed a significant colocalization with the formulation elaborated without protamine (R= 0.618 ± 0.035); when protamine was incorporated, colocalization with the cholera toxin decreased (R= 0.491 ± 0.046). The low levels of transferrin compared to cholera toxin levels indicate that this cell line uses barely endocytosis via clathrin.
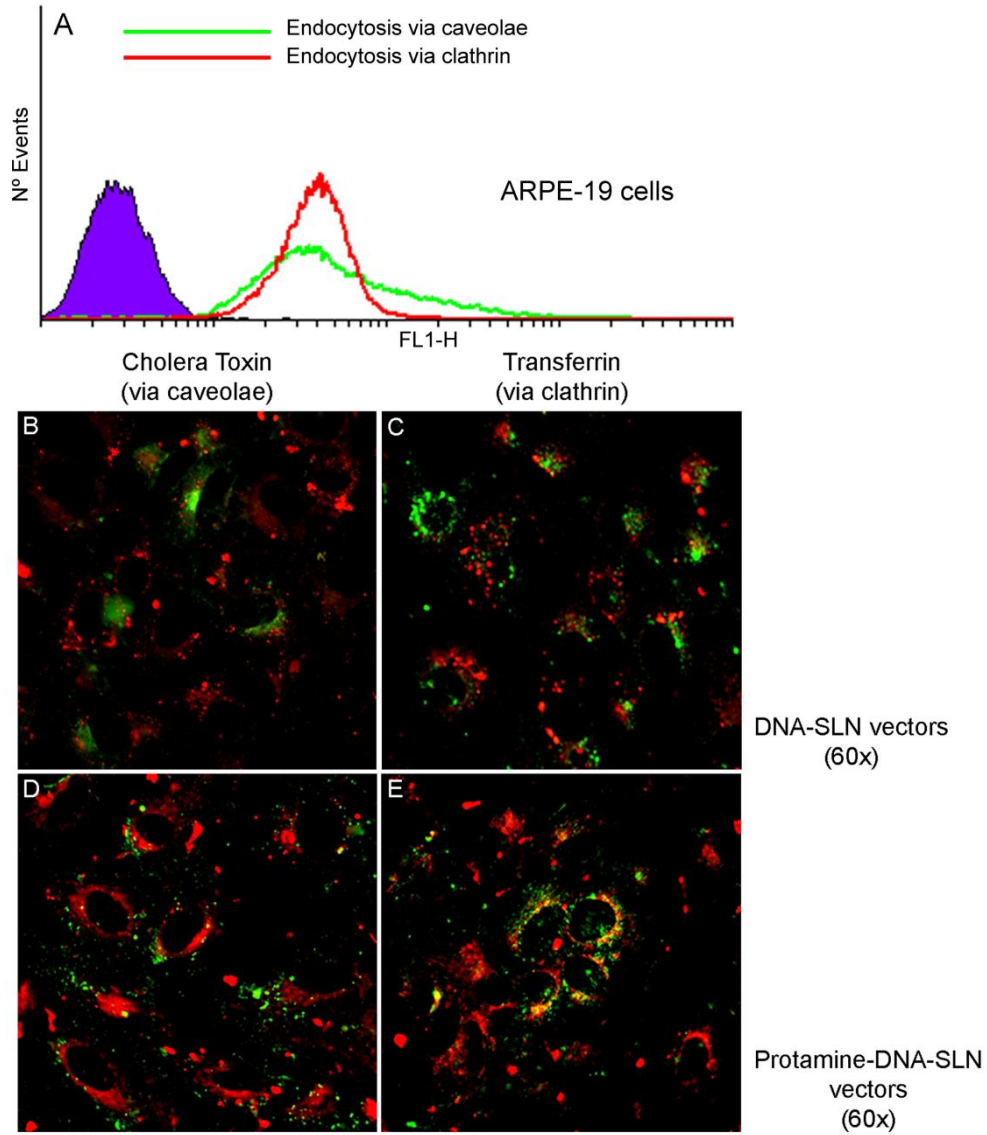
Figure 4. Presence of AlexaFluor 488-Cholera toxin and AlexaFluor 488-Transferrin quantified by flow citometry (A) and CLSM images of Nile Red-labeled DNA-SLN vectors (B-C) and protamine-DNA-SLN vectors (D-E) in ARPE-19 cells, with AlexaFluor 488-Cholera toxin (green) at 10 μg/ml, (B) and (D); or with AlexaFluor 488-Transferrin (green) at 50 μg/ml, (C) and (E). SLN to DNA ratio (w/w) was 5:1 and protamine to DNA ratio was 2:1.
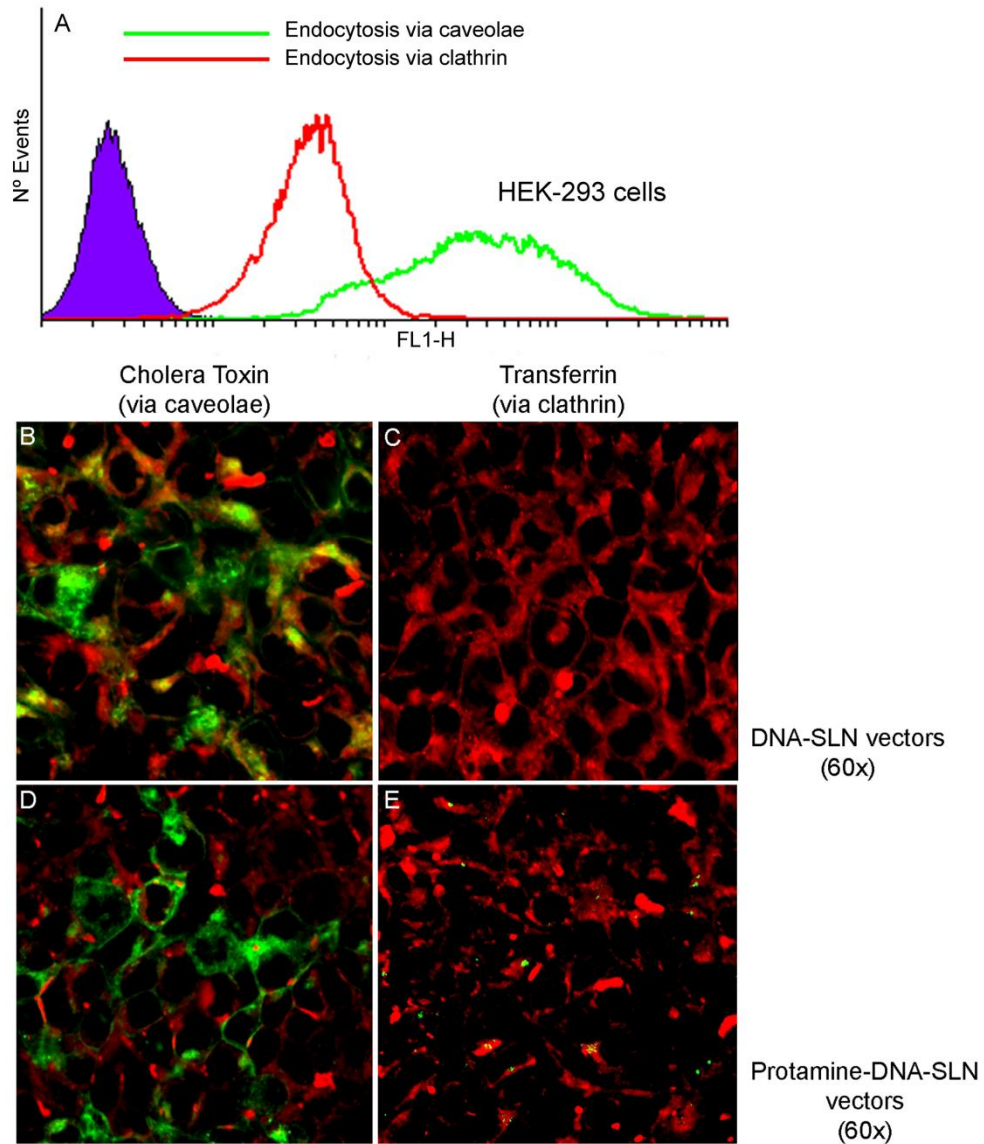
Figure 5. Presence of AlexaFluor 488-Cholera toxin and AlexaFluor 488-Transferrin quantified by flow cytometry (A) and CLSM images of Nile Red-labeled DNA-SLN vectors (B-C) and protamine-DNA-SLN vectors (D-E) in HEK-293 cells, with AlexaFluor 488-Cholera toxin (green) at 10 μg/ml (B) and (D); or with AlexaFluor 488-Transferrin (green) at 50 μg/ml (C) and (E). SLN to DNA ratio (w/w) was 5:1 and protamine to DNA ratio was 2:1.

3.6. Intracellular distribution of EMA-labelled DNA in culture cells

Figure 6 presents images captured by fluorescence microscopy from 4h to 72h after the addition of the vectors. When ARPE-19 cells were treated with DNA-SLN vector, red fluorescence appeared homogeneously distributed in the cytoplasm, but DNA was much more condensed and closer to the nucleus with protamine-DNA-SLN vector. However in HEK-293 cells, DNA appeared condensed and close to the nucleus with both vectors.
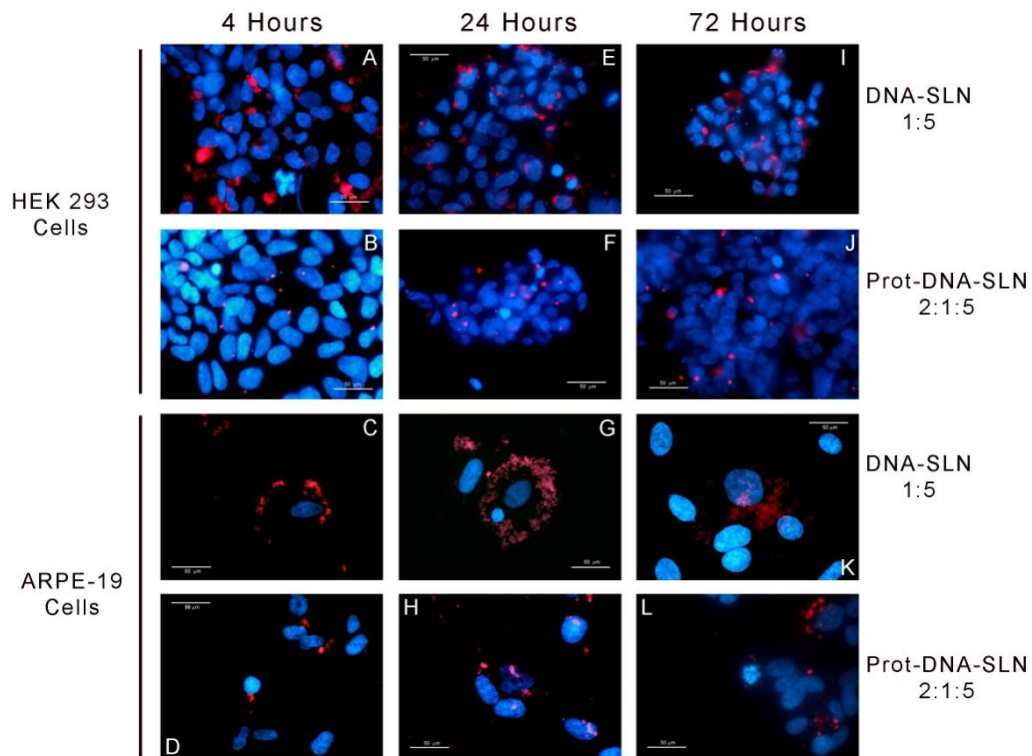
Figure 6. Fluorescence microscopy images of HEK-293 cells and ARPE-19 cells at different times after the addition of DNA-SLN vector or protamine-DNA-SLN vector. Cells were treated with Hoechst 33258 in order to detect the nucleus (blue) and vectors containing EMA-labeled pCMS-EGFP plasmid (red). The SLN to DNA ratio (w/w) was 5:1 and the protamine to DNA ratio 2:1.

4. DISCUSSION

The incorporation of NLS to non-viral vectors has been studied by several authors and they have shown improvement in transfection due to an effect on the nuclear membrane.[25,26,27] Protamine is a peptide widely used to improve lipofection due to its content in NLS. The enhancement of transfection efficacy with protamine is attributed to nuclear localization and stabilization against DNase degradation.[28] However, there is very little information describing its role in lipofection.[29] It is well-known that transfection efficiency of nanoparticular vectors is conditioned by cell line-dependent factors such as division rate, internalization pathway and intracellular trafficking.[30,31] Thus, in this work we evaluated the transfection efficiency of protamine-DNA-SLN vector and its correlation with those factors by using two different cell lines: ARPE-19 and HEK-293 cells.

Our results indicated that entry through clathrin-mediated endocytosis is necessary in order to achieve transfection when protamine-DNA complexes are incorporated on the SLN. In spite of the high DNA condensation capacity of protamine and its content in NLS, this does not always lead to an improvement in cell

[25] Kanazawa T, Takashima Y, Murakoshi M, Nakai Y, Okada H. Enhancement of gene transfection into human dendritic cells using cationic PLGA nanospheres with a synthesized nuclear localization signal. Int J Pharm. 2009;379:187-195.

[26] Hoare M, Greiser U, Schu S, Mashayekhi K, Aydogan E, Murphy M, et al. Enhanced lipoplex-mediated gene expression in mesenchymal stem cells using reiterated nuclear localization sequence peptides. J Gene Med. 2010;12:207-218.

[27] Shen Y, Peng H, Pan S, Feng M, Wen Y, Deng J, et al. Interaction of DNA/nuclear protein/polycation and the terplexes for gene delivery. Nanotechnology. 2010;21:045102-045113.

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[30] Douglas KL, Piccirillo CA, Tabrizian M. Cell line-dependent internalization pathways and intracellular trafficking determine transfection efficiency of nanoparticle vectors. Eur J Pharm Biopharm. 2008;68:676-687.

[31] Duan Y, Zhang S, Wang B, Yang B, Zhi D. The biological routes of gene delivery mediated by lipid based non-viral vectors. Expert Opin Drug Del. 2009;12:1351-1361.

transfection; although NLS may have a positive effect in the entry to the nucleus, they may impair the previous steps due to the multistage nature of the transfection process. Therefore, the influence of any new component involved on each step of transfection process should always be studied.

As expected, the capacity of protamine to condensate DNA depends on the protamine to DNA ratio. Figure 1A shows that a ratio of at least 2:1 is needed to bind all DNA. Gel electrophoresis (Figure 1B) also shows that the presence of protamine increased the DNA protection capacity of the DNA-SLN complexes. Another important issue to be considered is the ability of the nanoparticles to deliver the DNA. Figure 1C shows that the higher the amount of protamine in the vectors, the more difficult the release of plasmid is. The formulation prepared at a protamine:DNA:SLN ratio of 2:1:5 possesses the suitable characteristics to transfect because this formulation increases the protection capacity of the DNA-SLN vector without hampering the plasmid release. Furthermore, the inclusion of protamine modified neither particle size of the vectors nor their zeta potential and all formulations prepared had a particle size around 250 nm (S.D. = 43.20) and a zeta potential about +35 mV (S.D. = 2.40). Modifications of particle size depends on the balance between the ability of the peptide to precondense DNA, which would imply a reduction in size, while demanding greater space itself, which would cause an increase in size. Thus, a change in particle size does not always happen. In a previous study we carried out with SLN containing other cationic peptide,[5] the peptide did not modify the surface charge of the nanoparticles. This could be due the limitations associated to the photon correlation to measured zeta potential.[32] Moreover, changes in the amount of charges

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[32] Eliyahu H, Siani S, Azzam T, Domb AJ, Barenholz Y. Relationship between cehemical composition, physical propertis and transfection efficiency of polysaccharide-spermine conjugates. Biomaterials. 2006;27:1646-1655.

may not always imply modification in the disposition of charges at the surface of the nanoparticles.

Once the vectors were characterized, the transfection capacity and cell viability in ARPE-19 and HEK-293 cells were studied. ARPE-19 cells divide slowly and are a good "in vitro" model to evaluate transfection systems targeted to the treatment of retinal diseases. On the contrary, HEK-293 cells divide rapidly and are usually employed for "in vitro" transfection studies. In a previous work, we have shown different transfection capacity of SLN to transfect ARPE-19 and HEK-293 cells,[10] achieving significant higher transfection levels in HEK-293 cultures. In a later work,[5] we suggested that the passage to the nucleus may be the main limitation for transfection of retinal pigment epithelial cells.

The highest ARPE-19 positive level (Figure 2A) was reached with a 2:1 protamine to DNA ratio (29% cells transfected), according to the condensation, protection against DNase and delivery studies. On the one hand, at lower protamine to DNA ratios all the basic aminoacids of protamine may be consumed in the condensation of DNA, which results in the limitation of the recognition of NLS.[21] On the other hand, if the protamine to DNA ratio is too high, DNA may be too condensed, and its release is more difficult. It should be noted that the increase of the transfection level in ARPE-19 due to protamine (from 5% to 29% transfected cells) must be considered a relevant achievement, since difficulties for transfecting this cell line are well known.[33] Until now, no other paper in the literature has shown a similar transfection level in ARPE-19 cells with non-viral vectors. These results confirm the importance of optimizing the proportion of components in the design of formulations.

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[33] Bejjani RA, BenEzra D, Cohen H, Rieger J, Andrieu C, Jeanny JC, et al. Nanoparticles for gene delivery to retinal pigment epithelial cells. Mol Vis. 2005;17:124-132.

Unexpectedly, the incorporation of protamine to our vectors decreased transfection in HEK-293 cells (from 60% to 2% transfected cells) in a protamine concentration manner (Figure 2B). Taking into account that this cell line shows a high-division rate, nuclear entry should not be a limiting step in transfection. That is why we expected that the inclusion of protamine in the vectors might not affect or increase transfection in these cells. Our results suggest that protamine may also affect other critical limiting steps in the transfection process. Therefore, the whole process involved in transfection should be considered, in order to properly explain the mechanism of protamine action, and not only the nuclear effect.

Other authors have also used protamine with lipid formulations at different protamine:DNA ratios, and as in our study, protamine not always produced an increase in transfection. In these studies, the effect of protamine was not related to the internalization mechanism and intracellular trafficking of the vectors.[34,35]

In ARPE-19 cultures, the percentage of cells that captured vectors did not change ($p > 0.05$) with the presence of protamine in the formulations (77% for DNA-SLN vs. 72% for protamine-DNA-SLN at ratio w/w/w 2:1:5); this lack of correlation between cell internalization and transfection in cell lines with low division rate has been described by other authors.[36] In HEK-293 cells, protamine brought about a decrease of cell uptake from 75% to 45% when the formulation prepared at protamine to DNA ratio of 2:1 was studied. As in ARPE-19 cells, no correlation between uptake

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[34] Faneca H, Simões S, Pedroso de Lima MC. Association of albumine or protamine to lipoplex: enhancement of trasnfection and resistance to serum. J Gene Med. 2004;6:681-692.
[35] Vighi E, Montanari M, Rouzi B, Tossi G, Magli A, Leo E. Nuclear localization of cationic solid lipid nanoparticles containing Protamine as trasnfection promoter. Eur J Pharm Biopharm. 2010;76:384-393.
[36] Mannermaa E, Rönkkö S, Ruponen M, Reinisalo M, Urtti A. Long-lasting secretion of transgene product from differentiated and filter-grown retinal pigment epithelial cells after non viral gene transfer. Curr Eye Res. 2005;30:345-353.

and transfection was found, since a decrease of 30% in cell uptake caused an almost complete inhibition of transfection.

Endocytosis has been postulated as the main entry mechanism for non-viral systems. Various endocytosis mechanisms have been described to date: phagocytosis, pinocytosis, clathrin-mediated and caveolae/raft-mediated. Clathrin-mediated endocytosis leads to an intracellular pathway in which endosomes fuse with lysosomes, which degrade their content, whereas caveolae/raft mediated endocytosis avoids the lysosomal pathway and its consequent vector degradation.

In ARPE-19 cells, clathrin mediated endocytosis and caveolae mediated endocytosis are present in a similar extent. Both mechanisms are involved in the internalization process of the vectors. However, the protamine-containing vectors presented a higher colocalization with transferrin (R = 0.641 ± 0.039 for protamine-DNA-SLN vectors vs. R = 0.424 ± 0.124 for DNA-SLN vectors), which indicates that protamine induces a shift in the internalization mechanism from caveolae/raft to clathrin endocytosis. Since the internalization pathway conditions later steps, we investigated the intracellular disposition of vectors prepared with EMA-labelled DNA.

We observed that DNA was highly condensed and near the nucleus after the treatment of both cell lines with the formulation containing protamine (Figure 6). However, when the cells were treated with DNA-SLN vector (without protamine), the plasmid was broadly distributed in the cytoplasm. In ARPE-19 cells, which show a slow division rate, an active transport of DNA through the nuclear pores is necessary; otherwise the DNA stays longer in the cytoplasm. Protamine is an excellent DNA condenser which reduces the exposition to degradation by different cytoplasmic agents such as DNAses, and helps the transport of DNA to the nucleus. This justified

the high level of transfection with the formulation containing protamine. Additionally, it has been shown that DNA condensed by protamine is more accessible to intra-nuclear transcription.[21]

In HEK-293 cells, the evaluation of endocytosis markers by flow cytometry indicated that endocytosis via caveolae is much more active than via clathrin (Figure 5). The images obtained by confocal microscopy also showed that the DNA-SLN vector mainly enter by caveolae/raft-dependent endocytosis. The vector containing protamine also used caveolae/raft-dependent endocytosis but to a lesser extent as suggest the colocalization coefficients (R = 0.491 ± 0.046 for protamine-DNA-SLN vectors vs. R = 0.618 ± 0.035 for DNA-SLN vectors). When vectors enter the cells by caveolae/raft-dependent endocytosis, there is a lack of lysosomal activity and the vectors are localized around the nucleus. Figure 6 shows that DNA presents the same localization and the same condensation degree with both vectors. The lack of lysosomal activity makes difficult the release of DNA from the solid lipid nanoparticles.

Since lipid particles are physiologically degraded, we followed transfection during 7 days to discard that delivery of the DNA in the cytoplasm might be dependent on time. Seven days after the addition of vectors, transfection levels did not increase in any of the two cell lines.

In a previous study carried out by our group,[5] we improved cell transfection of ARPE-19 by using a cell penetration peptide (SAP) with solid lipid nanoparticles. This peptide presents lower capacity of condensation and protection of DNA than protamine, thus via caveolae is its optimum endocytic mechanism since it localizes the vectors around the nucleus and lysosomal activity is reduced. With protamine,

endocytosis via clathrin is necessary for transfection because of the lysosomal activity that facilitates the release of the complex protamine-DNA to reach the nucleus. Depending on the composition of the vector, the most appropriate internalization mechanism may be modulated. Therefore, the knowledge of the cellular entry of gene delivery systems is a crucial step in the field of gene therapy. Nevertheless, developing studies to understand the process of endocytosis and intracellular trafficking of gene carriers "in vivo" is still the real challenge for the nest years of gene delivery.[37]

In summary, our study shows that the disposition of protamine on the surface of the vectors affects their interaction with the cell membrane and, consequently, their entrance into the cell and their transfection efficacy. Protamine induced a 6-fold increase in the transfection capacity of SLN in retinal cells due to a shift in the internalization mechanism from caveolae/raft-mediated to clathrin-mediated endocytosis, which promotes the release of the protamine-DNA complexes from the SLN; afterwards, the transport of the complexes into the nucleus is favoured by the NLS of the protamine. On the contrary, protamine diminished transfection in HEK-293 cells, due to the decrease in the cell uptake of the vectors and to the difficulty of the protamine-DNA complexes to be released from the nanoparticles in absence of lysosomal activity, which is characteristic of via caveolae. This study reinforces the importance of the knowledge of the internalization mechanism and intracellular trafficking processes, which can be modulated by optimizing the formulations to better design more efficient non-viral vectors.

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DEXTRAN-PROTAMINE-SOLID LIPID NANOPARTICLES AS POTENTIAL VECTORS FOR THE TREATMENT OF X LINKED JUVENILE RETINOSCHISIS.

Authors

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ABSTRACT
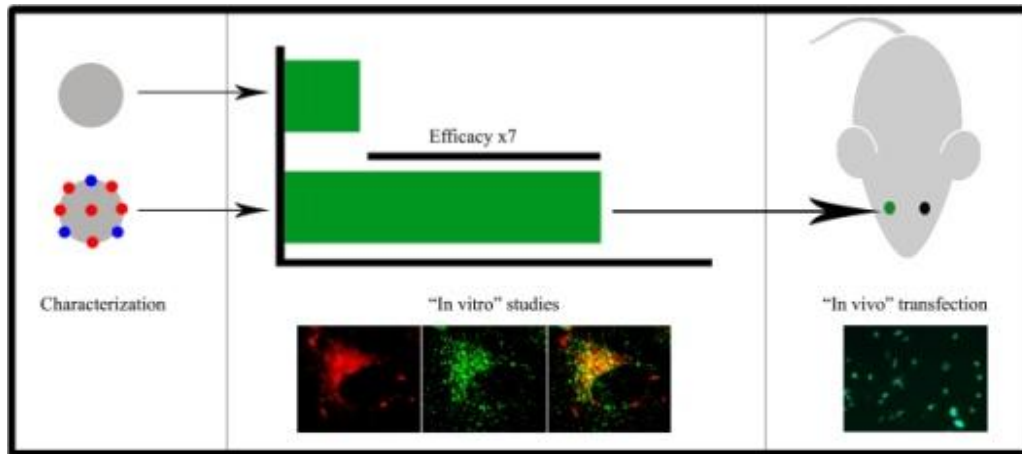
The aim of the present study was to analyze the potential application of non-viral vectors based on solid lipid nanoparticles (SLN) for the treatment of ocular diseases by gene therapy, specifically X linked juvenile retinoschisis (XLRS).

Vectors were prepared with SLN, dextran, protamine, and a plasmid (pCMS-EGFP or pCEP4-RS1). Formulations were characterized and the "in vitro" transfection capacity as well as the cellular uptake and the intracellular trafficking were studied in ARPE-19 cells. Formulations were also tested "in vivo" in Wistar rat eyes and the efficacy was studied by monitoring the expression of EGFP after intravitreal, subretinal and topical administration.

The presence of dextran and protamine in the SLN improved greatly the expression of retinoschisin and green fluorescent protein in ARPE-19. The nuclear localization signals (NLS) of protamine, its protection capacity and a shift in the internalization

mechanism from caveolae-mediated to clathrin-mediated endocytosis promoted by the dextran, justify the increase of transfection. After ocular administration of the dextran-protamine-DNA-SLN complex to the rat eyes, we detected the expression of EGFP in various types of cells depending on the administration route. Our vectors were also able to transfect corneal cells after topical application.

We have demonstrated the potential utility of our non-viral vectors loading XLRS1 plasmid and provided evidence for their potential application for the treatment of degenerative retina disorders as well as ocular surface diseases.

Keywords: Solid lipid nanoparticles; gene therapy; ocular disorders; intracellular trafficking; "in vivo" transfection

1. INTRODUCTION

Although there have been important advances in the treatment and prevention of eye disease over the past few decades, there are still many causes of vision loss for which there is no cure, and even with the best medical treatment, many persons must live with impaired vision. Gene therapy is considered a promising option for the treatment of hereditary but also acquired eye diseases. Its clinical potential was recently demonstrated by major improvements of visual function in first clinical trials with patients suffering from Leber`s congenital amaurosis.[1,2] Generally, the eye is a promising target for gene therapy due to its unique features such as easy accessibility as well as convenient and highly sensitive methods for monitoring even minor changes of visual function. The well-defined anatomy and immunoprivilege of the eye are also important advantages for gene therapy.[3] Due to its relative small size, effective treatment of the ocular tissues will require minor product concentrations while the diffusion from the eye into the circulation is limited.[4,5]

Inherited retinal degenerations have an estimated prevalence of 1:4,000 with a progressive and often untreatable course. [6] For example, X linked juvenile retinoschisis (XLRS) is a common cause of juvenile blindness in males with a

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prevalence of 1:5,000 to 1:25,000.[7] The application of gene replacement therapy in XLRS has been considered a promising therapeutic approach for this disease. Proof-of-concept was provided by several groups using viral delivery systems in retinoschisin-deficient mice.[8,9] However, viral vectors present important limitations due to immunogenicity and oncogenicity.[10] Moreover, there are evidences for the potential persistence of viral vectors in brain after intravitreal injection.[11] These drawbacks have motivated the development of non-viral delivery systems.

In previous studies we have developed non-viral vectors based on cationic lipids and more precisely on solid lipid nanoparticles (SLN).[12,13] These SLN showed capacity to transfect "in vitro" retinal pigment epithelium cells (RPE cells), used as a model for inherited retinal diseases.[14] In an effort to expand our previous work, the aim of the present study was to analyze the potential application of solid lipid nanoparticle-based non-viral vectors for the treatment of ocular diseases, specifically XLRS. With this goal in mind, we measured the protein produced (green fluorescence protein or retinoschisin) after "in vitro" transfection of retinal cells (ARPE-19 cell

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[11] Provost N, Le Meur G, Weber M, Mendes-Madeira A, Povedin G, Cherel Y, et al. Biodistribution of rAAV vectors following intraocular dministration: evidence for the presence and presistence of vector DNA in the optic nerver and in the brain. Mol Ther. 2005;11:275-583.

[12] del Pozo-Rodríguez A, Pujals S, Delgado D, Solinís MA, Gascón AR, Giralt E, et al. A proline-rich peptide improves cell transfection of solid lipid nanoparticles-based non viral vectors. J Control Release. 2009;133:52-59.

[13] Delgado D, del Pozo-Rodríguez A, Solinís MA, Gascón AR. Understanding the mechanism of protamine in solid lipid nanoparticle-based lipofection: the importance of the entry pathway. Eur J Pharm Biopharm. 2011. In press.

[14] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles for retinal gene therapy: Transfection and intracellular trafficking in RPE cells. Int J Pharm. 2008;360:177-183.

line) with the vectors containing the plasmids that codify those proteins. We also followed the internalization of our vectors into the cells, their trafficking inside cytosol and the transport of plasmids to the nucleus in retinal cells. Following the "in vitro" characterization, the vectors were administered to rat eyes and the efficacy was studied by monitoring the expression of the enhanced green fluorescent protein.

2. MATERIALS AND METHODS

2.1. Materials

Precirol® ATO 5 was provided by Gattefossé (Madrid, Spain). 1,2-Dioleoyl-3-Trimethylammonium-Propane Chloride Salt (DOTAP) was provided by Avanti Polar Lipids, Inc. Deoxyribonuclease I (DNase I), lauryl sulphate sodium (SDS), Triton X-100, Isopropyl β-D-1-thiogalactopiranoside (IPTG), Nile Red, protamine sulphate salt from Salmon (Grade X), dextran (Mn=3260), 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) and normal goat serum were acquired from Sigma-Aldrich (Madrid, Spain). Tween 80 was purchased from Vencaser (Bilbao, Spain), and dichloromethane from Panreac (Barcelona, Spain). Immobilized D-galactose Gel was provided by Pierce (Madrid, Spain).

Plasmid pCMS-EGFP, which encodes the enhanced green fluorescent protein (EGFP), was purchased from BD Biosciences Clontech (Palo Alto, California, US) and amplified by Dro S.L. (San Sebastián, Spain). Plasmid pCEP4-RS1, which encodes the retinoschisin protein, was prepared in the Institute of Human Genetics, University of Regensburg (Regensburg, Germany).

Western Blot and gel electrophoresis materials were acquired from Bio-Rad and Sigma-Aldrich (Madrid, Spain).

Cell culture reagents were purchased from LGC Promochem (Barcelona, Spain). Antibiotic Normocin™ was acquired from InvivoGen (San Diego, California, US). BD Viaprobe kit was provided by BD Biosciences (Belgium).

AlexaFluor488-Cholera toxin and AlexaFluor488 Transferrin were provided by Molecular Probes (Barcelona, Spain), and Fluoromount G by SouthernBiotech (Coultek, España).

Monoclonal antibody RS1-3R10 was produced and provided from Institute of Human Genetics, University of Regensburg, Regensburg, Germany). [15] Secondary antibody AlexaFluor® 488 goat anti-rabbit IgG were provided by Invitrogen (Barcelona, Spain).

Wistar rats weighing 100-150 g (5 weeks of age) were purchased from Harlam Interfauna Ibérica S.L. (Barcelona, Spain). Ketolar® was provided by Pfizer (NY, USA), Rompun® by Bayer (Germany) and Colircusi tropicamida 1%® and Colircusi Anestésico Doble® by Alcon-Cusi (Barcelona, Spain). Hamilton syringes were purchased from Hamilton Company (Reno NV, USA) and fluorescent mounting media Vectashield from Vector Laboratories (USA).

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[15] Molday LL, Hicks D, Sauer CG, Weber BH, Molday RS. Expression of X-linked retinoschisis protein RS1 in photoreceptor and bipolar cells. Invest Ophtalmol Vis Sci. 2001;42:816-825.

2.2. Cell cultures

"In vitro" assays were performed with retinal pigment epithelial (ARPE-19) cells obtained from the American Type Culture Collection (ATCC). ARPE-19 cells were maintained in Dulbecco's Modified Eagle's Medium-Han's Nutrient Mixture F-12 (1:1) medium (D-MEM/F-12) supplemented with 10% heat-inactivated fetal calf serum and 1% Normocin™ antibiotic solution. Cells were incubated at 37 ºC under 5% CO2 atmosphere and subcultured every 2 to 3 days using trypsin-EDTA.

2.3. Production of the vectors

2.3.1. Preparation of SLN

The SLN were produced by a solvent emulsification-evaporation technique previously described by del Pozo-Rodríguez et al. 2007.[16] Briefly, Precirol® ATO 5 was dissolved in dichloromethane (5% w/v), and then emulsified by sonication (Branson Sonifier 250, Danbury) for 30 seconds at 50 W in an aqueous phase containing DOTAP (0.4% w/v) and Tween 80 (0.1% w/v). The organic solvent was removed by evaporation and SLN suspension was formed upon solidification of the Precirol® ATO 5. The SLN were washed by centrifugation (3,000 rpm, 20 minutes, x 3) using Millipore (Madrid, Spain) Amicon® Ultra centrifugal filters (100,000 MWCO).

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[16] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles: Formulation factors affecting cell transfection capacity. Int J Pharm. 2007;339:261-268.

2.3.2. Preparation of the dextran-protamine-DNA complexes

Firstly, an aqueous solution of dextran was mixed with and aqueous solution of protamine to form the dextran-protamine complexes at w/w ratios of 0.5:2, 1:2 and 2:2. Then, pCMS-EGFP plasmid was bound to the dextran-protamine complexes to set dextran-protamine-DNA ratios of 0.5:2:1, 1:2:1 and 2:2:1 (w/w/w). The resulting complexes were diluted in Milli-Q™ water up to a final concentration of 0.03 µg DNA/µL, and then subjected to electrophoresis on a 1% agarose gel (containing ethidium bromide for visualization) for 30 min at 120 V. The bands were observed with an Uvitec Uvidoc D-55-LCD-20M Auto transilluminator.

2.3.3. Preparation of the vectors

The DNA-SLN vector was obtained by adding the DNA plasmid to an aqueous suspension of SLN. The SLN to DNA ratio, expressed as the ratio of DOTAP to DNA (w/w), was fixed at 5:1 with pCMS-EGFP plasmid. Regarding pCEP4-RS1 plasmid, ratios 5:1 and 6:1 were compared.

The dextran-protamine-DNA-SLN vector was prepared by mixing the dextran-protamine-DNA complexes with SLN suspension under agitation for 30 minutes. Interaction between free negative charges of DNA and positive charges of SLN led to the formation of the vectors, in which dextran-protamine-DNA complexes are adsorbed on the nanoparticle surface.

Sizes of SLN, DNA-SLN and dextran-protamine-DNA-SLN (1:2:1:5) vectors were determined by photon correlation spectroscopy (PCS). Zeta potentials were

measured by laser Doppler velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). All samples were diluted in 0.1 mM NaCl (aq.).

2.4. DNAse protection study and DNA plasmid release study

DNase I was added to DNA-SLN and dextran-protamine-DNA-SLN vectors to a final concentration of 1 U DNase I/2.5 µg DNA, and the mixtures were then incubated at 37 ºC for 30 min; afterwards, 2% SDS solution was added to the samples to a final concentration of 1% to release the DNA from the SLN. The samples were then analyzed by electrophoresis on agarose gel (described above), and the integrity of the DNA in each sample was compared to a control of untreated DNA.

2.5. "In vitro" transfection assays

2.5.1. Vectors containing pCMS-EGFP plasmid

For transfection with pCMS-EGFP plasmid, ARPE-19 cells were seeded on 12 well plates at a density of 30,000 cells per well and allowed to adhere overnight. Vectors were diluted in HBS and added to the cell cultures. In all cases, 2.5 µg of DNA were added. Cells were incubated with the vectors at 37ºC, and after 4 h, the medium containing the complexes in the wells was refreshed with 1 mL of complete medium. The cells were then allowed to grow for another 72 h. Transfection efficacy was quantified at 12 h, 24 h, 48 h, 72 h and one week. As control, the DNA-SLN vector whose transfection ability was evaluated in previous works was also assayed.[12,14]

Transfection evaluation by flow cytometry.

At the point times, the cells were washed once with 300 µL of PBS, and then detached with 300 µL of 0.05% trypsin-EDTA. The cells were then centrifuged at 1,500 x g, and the supernatant was discarded. After resuspension in PBS, cells were directly introduced into a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, California, US). For each sample, 10,000 events were collected. Transfection efficacy was quantified by measuring the fluorescence of EGFP at 525 nm (FL1). For cell viability measurements, the BD Via-Probe kit was employed. This reagent was used to exclude dead cells from the analysis. The reagent (5 µL) was added to each sample, and after 10 minutes of incubation, the fluorescence corresponding to dead cells was measured at 650 nm (FL3).

2.5.2. Vectors containing pCEP4-RS1 plasmid

Immunochemical detection of retinoschisin.

ARPE-19 cells were seeded in coverslips and allowed to adhere overnight. The vectors were diluted in HBS and added to the cell cultures. In all cases, 2.5 µg of DNA were added. The cells were incubated with the vectors at 37 ºC, and after 4 h, the medium containing the complexes in the wells was refreshed with 1 mL of complete medium. After 72 h, the samples were permeabilized and blocked with PBS containing 0.2% Triton X-100 and 10% normal goat serum for 20 min and labeled overnight with the RS1-3R10 antibody diluted in PBS containing 0.1% Triton X-100 and 2.5% normal goat serum. Samples were rinsed in PBS and labeled for 1 h with Alexa Fluor® 488 goat anti-rabbit IgG. ARPE-19 cells were also treated with DAPI nuclear stain and images were captured with an inverted microscopy equipped with an attachment for fluorescent observation (model EclipseTE2000-S, Nikon).

Quantify of secreted retinoschisin.

ARPE-19 cells were seeded on 100 mm culture dishes and allowed to adhere overnight and to reach 80-90% cell confluence. The vectors were diluted in HBS and added to the cell cultures. Ten μg of DNA were added and the cells were incubated with the vectors at 37 °C during 72 h. Secreted proteins were then extracted from cells media by means of acetone precipitation and centrifugation. Extracted proteins were diluted in TBS (20mN Tris-HCl and 150 mM NaCl; pH=7.5). The samples were incubated with agarose conjugated with galactose beads (1:1 packed beads to TBS buffer) overnight at 4°C. The beads were washed three times with a TBS buffer and the retinoschisin was dissociated from the beads by incubating with TBS containing Isopropyl β-D-1-thiogalactopiranoside (IPTG) for 30 min at room temperature. The retinoschisin concentration was determined by the Bradford Method.[17]

2.6. Cellular uptake of non-viral vectors

Entry of vectors to the cells was studied quantitatively by flow cytometry. For this purpose SLN were labeled with the fluorescent dye Nile Red (λ = 590nm) according to a previously reported method (Borgia et al., 2005). SLN were prepared by the emulsification-evaporation technique described above, in which the Nile Red was incorporated into the dichloromethane.

Two hours after the addition of the labeled vectors, cells were washed three times with PBS and detached from plates. Cells incorporating either DNA-SLN or dextran-protamine-DNA-SLN vectors were quantified by flow cytometry at 650 nm (FL3). For each sample, 10,000 events were collected.

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[17] Dyka FM, Wu WHW, Pfeifer TA, Molday LL, Grigliatti TA, Molday RS. Characterization and purification of the discoidin domain containing protein retinoschisin and its interaction with galactose. Biochemistry. 2008;47:9098-9106.

2.7. Colocalization assay

The endocytic processes involved in the internalization of the non-viral systems were analyzed qualitatively by colocalization studies with AlexaFluor488-Cholera Toxin and AlexaFluor488-Transferrin, which are markers of caveolae/raft mediated endocytosis and clathrin mediated endocytosis, respectively.

For the confocal laser scanning microscopy (CLSM) study, cells were seeded in coverslips containing plates and co-incubated for 2 hours with Nile Red labeled vectors and either AlexaFluor488-Cholera toxin (10 μg/ml) or AlexaFluor488-Transferrin (50 μg/ml). After incubation, the medium was removed and cells were washed with PBS and fixed with paraformaldehyde 4%. We had previously checked that paraformaldehyde did not interact with the fluorescence of Nile Red or EGFP.

Preparations were mounted on Fluoromount G and afterwards air-drying images were obtained with an Olympus Fluoview FV500 confocal microscope, using sequential acquisition to avoid overlapping of fluorescent emission spectra. CLSM images were captured in the General Service of Analytical Microscopy and High Resolution in Biomedicine of the University of the Basque Country (UPV-EHU).

Colocalization results were estimated by means of Manders Overlap Coefficient (R), where $0.6 \leq R \leq 1.0$ indicates colocalization (overlap of the signals).[18]

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[18] Zinchuk V, Zinchuk O. Quantitative colocalization analysis of confocal fluorescence microscopy images. Curr Protoc Cell Biol. 2008;39:4.19.1-4.19.16.

2.8. "In vivo" study in rats

The animal experiments followed the European Union regulations for the use of animals in research; the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research; and the guidelines published by the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals). The animal protocol was approved by the Institutional Animal Care and Use Committee of the University Miguel Hernández.

The surgical procedures used for the transplantation and administration of vectors without plasmid (control) or dextran-protamine-DNA-SLN complexes in the retina have been described elsewhere.[19,20] Briefly, rats kept in a 12 h light-dark cycle, were anaesthetized with an intraperitoneal injection of a mixture of ketamine (70 mg/kg Ketalar®, Pfizer, Alcobendas, Madrid, Spain) and xylazine (10 mg/kg Rompun®, Bayer, Kiel, Germany) in 0.1 ml saline.

Next, their pupils were dilated with one drop of 1% tropicamide solution (Colircusi tropicamida®, Alcon, Barcelona, Spain) and the cornea was anesthetized with a drop of 4% oxybuprocaine and 1% tetracaine (Colircusi Anestesico Doble®, Alcon, Barcelona, Spain).

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[19] Nour M, Quiambao AB, Peterson WM, Al-Ubaidi MR, Naash MI. P2Y(2) receptor agonist INS37217 enhances functional recovery after detachment caused by subretinal injection in normal and rds mice. Invest Ophthalmol Vis Sci. 2003;44:4505-4514.

[20] Jiang C, Moore MJ, Zhang X, Klassen H, et al. Intravitreal injections of GDNF-loaded biodegradable microspheres are neuroprotective in a rat model of glaucoma. Mol Vis. 2007;13:1783-1792.

2.8.1. Intravitreous administration

Intravitreal injection was performed under general anesthesia using a Zeiss operating microscope (Zeiss, OPMI). A 30-gauge hypodermic needle was used to perforate the sclera 1.5 mm behind the limbus. The hypodermic needle was then withdrawn, and a 33-gauge blunt needle (Hamilton Co, Reno, NV) was inserted through the hole and advanced into the anterior chamber avoiding the iris and lens.

The needle was angled to point slightly nasally and guided posteriorly into the eye. The lens was displaced medially as the needle was advanced through the vitreous cavity toward the retinal surface. Once the tip of the needle had reached the desired location, 3 µl of dextran-protamine-DNA-SLN solution (0.1 µg plasmid DNA) were injected into the vitreous cavity of the left eye. Care was taken not to damage the lens.

Following intraocular injections, the needle was held in place for one minute and withdrawn slowly. In addition, paracentesis was simultaneously performed to relieve pressure and thereby prevent reflux.

The right eye was kept as control. After the injection, one drop of ofloxacin ophthalmic solution (Exocin®, Allergan, Barcelona, Spain) was applied to both eyes.

Finally the retinas were examined funduscopically with a stereomicroscope (Leitz, Wetzlar, Germany) after the injection, and animals with retinal bleeding or lens injury following the injection procedure were excluded from the study.

2.8.2. Subretinal administration

The procedure was similar to the intravitreous injection and was performed under general anesthesia using a Zeiss operating microscope (Zeiss, OPMI). A 30-gauge hypodermic needle was used to perforate the sclera. With a 30-gauge needle (Hamilton, Reno, NV), the dextran-protamine-DNA-SLN vector was injected into the superior subretinal space of the left eye.

The needle passed through the sclera and then 1-2 µL of the dextran-protamine-DNA-SLN complexes were injected (0.03-0.06 µg plasmid DNA).

After the injection, one drop of ofloxacin ophthalmic solution (Exocin®, Allergan, Barcelona, Spain) was applied as antibiotic to the eye.

The retinas were examined funduscopically with a stereomicroscope (Leitz, Wetzlar, Germany) after the injection, and eyes showing massive subretinal hemorrhage, vitreous hemorrhage, or large retinal detachments were excluded.

2.8.3. Topical administration

For the "in vivo" study, rat corneas were treated with dextran-protamine-DNA-SLN complexes by simple instillation of drops (10 µL, a single time, 3 µg plasmid DNA). To avoid possible interactions neither antibiotics nor any other ophthalmic solutions were used after the administration.

2.8.4. Evaluation of gene expression

To evaluate the yield of vector transfer efficiency "in vivo", the expression of the green protein was observed 72 hours after the intravitreal and subretinal injections, and 72 and 120 hours after the topical instillation in the corneas.

Animals were killed with an intraperitoneal overdose of sodium pentobarbital. The eyes were enucleated and fixed overnight in 4% paraformaldehyde in 0.1M phosphate-buffered saline (PBS).

Then the eyes were washed with PBS, the corneas excised and the retinas isolated. Flatmounted preparations of the retina, including the RPE layer, and cornea were examined and photographed by fluorescence microscopy (Model AX70, Olympus).

2.9. Statistical analysis

Results are reported as mean values (SD = standard deviation). Statistical analysis was performed with SPSS 17.0 (SPSS®, Chicago, IL, USA). Normal distribution of samples was assessed by the Shapiro–Wilk test, and homogeneity of variance, by the Levene test. The different formulations were compared with ANOVA and student´s t test, whereby differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. Production of the vectors

Dextran, protamine and pCMS-EGFP plasmid were combined at ratios of 0.5:2:1, 1:2:1 and 2:2:1, respectively, and the resulting complexes were subjected to electrophoresis on an agarose gel (Figure 1A). Results indicate that DNA is fully bound in the complexes at all indicated dextran and protamine proportions.

The DNA-SLN vector and the dextran-protamine-DNA-SLN vector showed particle sizes between 200 and 270 nm, and surface charge of about +35 mV, irrespective of the plasmid used, i.e. pCMS-EGFP or pCEP4-RS1. No differences in particle size and zeta potential between DNA-SLN and dextran-protamine-DNA-SLN formulations were detected (p>0.05).

3.2. "In vitro" resistance against DNase and SDS-induced release of DNA from vectors

The protection capacity of the DNA-SLN and dextran-protamine-DNA-SLN vectors and the release of DNA from them were studied by analyzing the integrity of DNA in agarose gel electrophoresis after treatment with DNase I (Figure 1B) and SDS (Figure 1C). These figures show the ability of both vectors to preserve and release the pCMS-EGFP plasmid they transport. When the vectors were prepared with the pCEP4-RS1 plasmid, a DNA:SLN ratio of 1:6 showed a higher protection capacity than the 1:5 ratio without hampering the plasmid release (Figure 2).
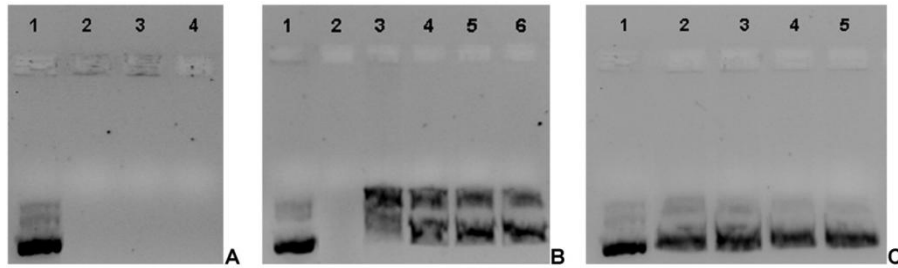
Figure 1A. Binding efficiency of DNA pCMS-EGFP plasmid at different dextran-protamine-DNA ratios (w/w/w) studied by agarose gel electrophoresis. Lane 1= free DNA; lane 2= 0.5:2:1; lane 3= 1:2:1; lane 4= 2:2:1. **Figure 1B.** Protection of DNA by dextran-protamine-DNA-SLN vectors from DNase digestion at different dextran-protamine-DNA-SLN ratios (w/w/w/w). Vectors were treated with DNase I. Lane 1= free DNA; lane 2= DNase-treated free DNA; lane 3= DNA-SLN vectors; lane 4= 0.5:2:1:5; lane 5= 1:2:1:5; lane 6= 2:2:1:5. **Figure 1C.** Release of DNA by dextran-protamine-DNA-SLN vectors from DNase digestion at different dextran-protamine-DNA-SLN ratios (w/w/w/w). Vectors were treated with SDS. Lane 1= free DNA; lane 2= DNA-SLN; lane 3= 0.5:2:1:5; lane 4= 1:2:1:5; lane 5= 2:2:1:5;

A. Ratio DNA:SLN 1:5

B. Ratio DNA:SLN 1:6
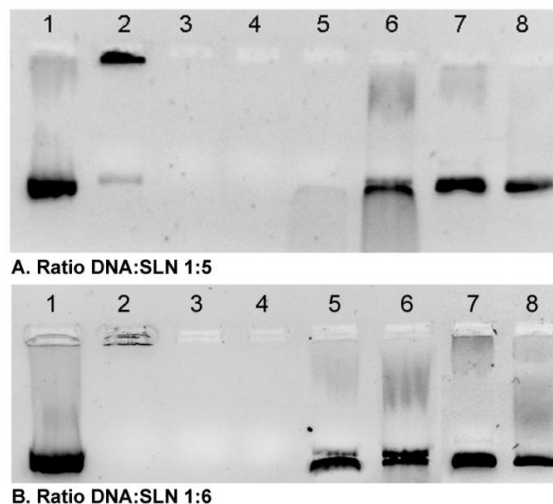
Figure 2. DNase protection and pCEP4-RS1 binding and release ability from complexes.
Figure 2A. Lane 1= free DNA; lane 2= DNA-SLN; lane 3= dextran-protamine-DNA-SLN; lane 4= free DNA with DNase; lane 5= DNA-SLN with DNase; lane 6= dextran-protamine-DNA-SLN with DNase; lane 7= DNA-SLN with SDS; lane 8= dextran-protamine-DNA-SLN. The SLN to DNA ratio, expressed as the ratio of DOTAP to DNA (w/w), was fixed at 5:1; dextran-protamine solution was prepared at the ratio (w/w) 1:2. **Figure 2B**. Lane 1= free DNA; lane 2= DNA-SLN; lane 3= dextran-protamine-DNA-SLN; lane 4= free DNA with DNase; lane 5= DNA-SLN with DNase; lane 6= dextran-protamine-DNA-SLN with DNase; lane 7= DNA-SLN with SDS; lane 8= dextran-protamine-DNA-SLN with SDS. The SLN to DNA ratio, expressed as the ratio of DOTAP to DNA (w/w), was fixed at 6:1; dextran-protamine solution was prepared at the ratio (w/w) 1:2.

3.3. Transfection and cell viability studies in ARPE-19 cells

Plasmid pCMS-EGFP

The vectors prepared at different dextran-protamine ratios were assayed to study their capacity for cell transfection in ARPE-19 cells. We had previously showed that dextran-protamine-DNA complexes were not able to transfect cells (data not shown). As observed in Figure 3A (bars), transfection levels 72 hours after addition of vectors to the ARPE-19 cells were at a maximum (44% EGFP positive cells) when dextran-protamine-DNA-SLN vectors were used at a 1:2:1:5 ratio, whereas the DNA-SLN vector resulted in only 7% transfected cells ($p<0.01$). Cell viability (line in Figure 3A) obtained with the vectors containing dextran and protamine was significantly higher than viability obtained with the DNA-SLN vector ($p<0.01$).

Figure 3. (A)Transfection (bars) and cell viability (line) for each formulation assayed in ARPE-19 cells at 72 hours; the SLN to DNA ratio (w/w) was 5:1 in all cases, and the dextran-protamine to DNA ratio varied from 0.5:2:1 to 2:2:1; (B) transfection of non-viral vectors in ARPE-19 cells in a time dependence. The SLN to DNA ratio (w/w) was 5:1 and dextran-protamine to DNA ratio was 1:2:1 (B). Error bars represent S.D. (n = 3). **$p<0.01$ respect to DNA-SLN formulation. [a] $p<0.01$ respect to all formulations.
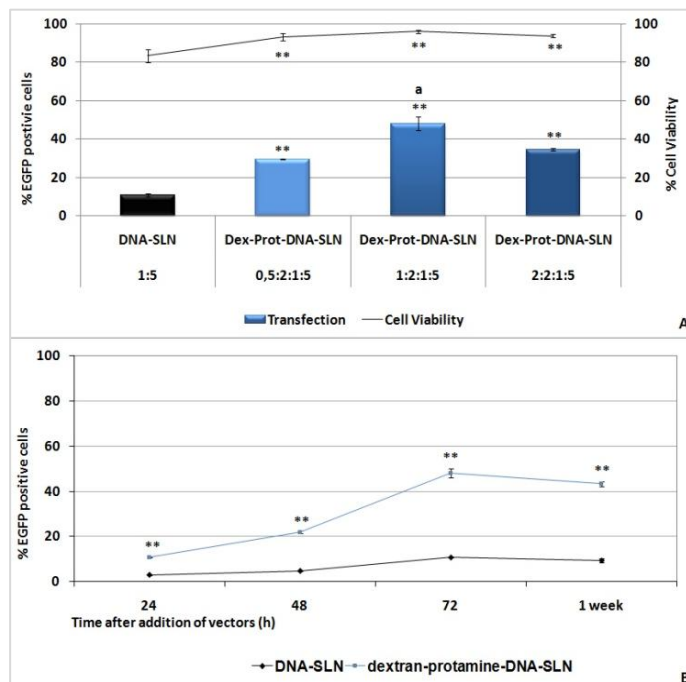
Subsequently, the transfection capacity of the most effective formulation (dextran-protamine-DNA-SLN at 1:2:1:5 ratio) and control formulation (DNA-SLN at ratio 1:5) was studied in a time dependence (Figure 3B). Transfection was detected from 24 h and progressively increased with both formulations, but at every time point dextran-protamine-DNA-SLN formulations showed higher efficacy ($p<0.01$).

Plasmid pCEP4-RS1

Retinoschisin present in cytoplasm 72 hours after transfection of ARPE-19 cells with pCEP4-RS1 plasmid was detected by immunochemistry. Figure 4 shows the protein expressed (green) after treating cells with DNA-SLN (Figure 4B) and dextran-protamine-DNA-SLN vectors (Figure 4A). Both vectors were able to transfect pCEP4-RS1. Cell nucleuses were labeled with DAPI (blue).

Figure 4C shows the levels of retinoschisin secreted by ARPE-19 cells 72 hours post-transfection. The presence of dextran and protamine in the nanoparticles induced a significant higher expression of retinoschisin ($p<0.01$). These results are consistent with those obtained with pCMS-EGFP.
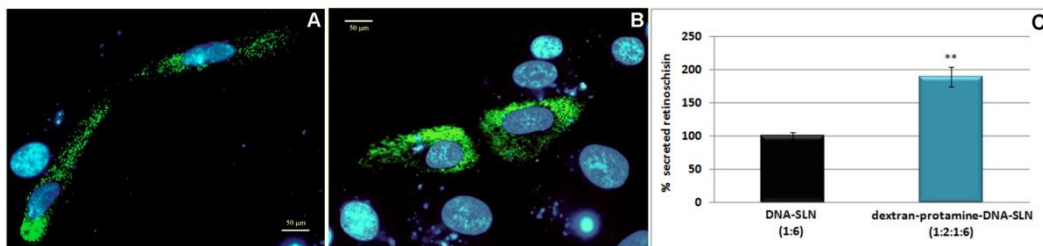
Figure 4. Detection of retinoschisin expressed in ARPE-19 cells by fluorescence microscopy after transfection with dextran-protamine-DNA-SLN vector (1:2:1:6) (A) and DNA-SLN vector (1:6) (B), and quantify of secreted retinoschisin (C). Green: retinoschisin labeled with Alexa Fluor 488. Blue: DAPI nuclear stain. Bar= 50 μm. The amount of retinoschisin (μg) per ml secreted by ARPE-19 cells treated with DNA-SLN vector was set to 100%.
** $p<0.01$ respect to DNA-SLN vectors.

3.4. Cellular uptake of non-viral vectors

No differences in cell uptake were detected between the two formulations (DNA-SLN and dextran-protamine-DNA-SLN vectors). The presence of dextran and protamine in the vectors did not modify their capacity to entry in ARPE-19 cells (Figure 5).
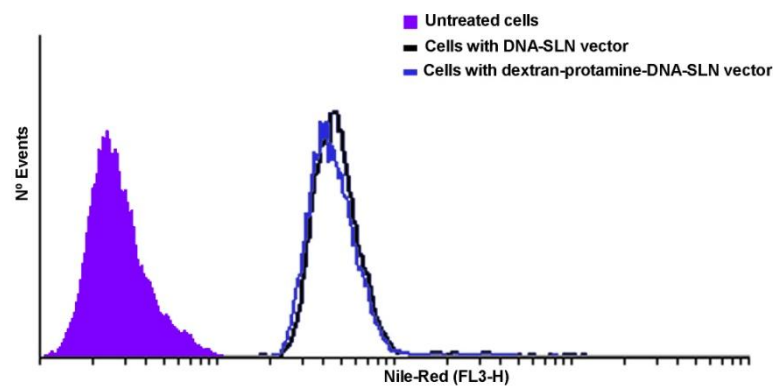
Figure 5. Flow cytometry analysis of cellular uptake study of DNA-SLN vector and dextran-protamine-DNA-SLN vector in ARPE-19 cells. Vectors were labeled with Nile Red.

3.5 Colocalization assay

The uptake mechanism of the two vectors in ARPE-19 cells was studied by using a confocal laser scanning microscopy (CLSM). Colocalization studies were carried out with AlexaFluor488-labelled Transferrin and Cholera Toxin, the markers of endocytosis via clathrin and via caveolae, respectively (Figure 6).

Both markers were found in the cells, suggesting that the two mechanisms of endocytosis are present in this cell line. The most significant finding was a higher

colocalization with transferrin when the vector containing protamine and dextran was added to the cells (yellow colour in figure 6D). This result is confirmed by the Mander`s Overlap coefficient (Table 1); the higher value of this coefficient (0,78) was achieved with the formulation dextran-protamine-DNA-SLN and transferrin (values close to 1 indicate high colocalization). Figure 6E shows magnifications of Figures 6C and 6D, featuring colocalization between the vector and the clathrin marker and the lack of colocalization with the caveolae marker. These results indicate that when dextran and protamine are added to the SLN, the endocytosis via clathrin is the predominant mechanism of cell internalization.

ARPE-19 Cells

	Endocytosis Via Caveolae		**Endocytosis Via Clathrin**	
	DNA-SLN	**Dex-Prot-DNA-SLN**	**DNA-SLN**	**Dex-Prot-DNA-SLN**
R	0.535 ∓ 0.174	0.378 ∓ 0.037	0.424 ∓ 0.124	0.780 ∓ 0.050

R = Mander's Overloap coefficient; values from 1 (high colocalization) to 0 (low colocalization)

Table 1. Coefficients of colocalisation analysis in ARPE-19 cells. **R**: Mander's Overlap coefficient. Values from 1 to 0; values more than 0.6 indicates high colocalization.

3.6. Protein expression after ocular administration

In order to explore the "in vivo" potential of the developed SLN complexes in ocular gene therapy, the dextran-protamine-DNA-SLN vector was loaded with plasmid encoding enhanced green fluorescent protein (pCMS-EGFP) and injected into the retina by different administration routes. Our data show that pCMS-EGFP can be successfully delivered and expressed in the eye (Figure 7). The level of expression in the eye varied depending on the location of the intraocular injection.

Thus, after intravitreal injection, high levels of gene expression were found mainly at the retinal ganglion cells (Figure 7A). Only a modest expression was found in the remaining layers of the retina and very little expression was detected in the retinal pigment epithelium (RPE). In contrast, after subretinal injection, expression was substantially higher in the RPE layer (Figure 7B). Furthermore, we also found some expression in photoreceptors and the outer nuclear layer (ONL).

To further assess the "in vivo" efficacy of the dextran-protamine-DNA-SLN complexes for the delivery of nucleic acids into ocular tissue, a solution of the complexes was instilled onto rat corneas. Gene expression was visible 72h post-administration (Figure 8). Taken together, the vectors were able to interact with corneal cells and penetrate through the corneal epithelium. The EGFP expression lasted at least 5 days (Figure 8C) and was located mainly inside the cells suggesting a transcellular mechanism of transport.

No green fluorescence was detected in the samples from rats treated with vectors without plasmid. Evidence of toxicity was not detected in the animals, irrespective of the formulation administered.

4. DISCUSSION

In spite of recent advances in the field of viral gene therapy for the treatment of degenerative ocular diseases, a safe and highly efficient alternative delivery system is still needed. Therapy with viral vectors improved the visual function in patients with Leber`s congenital amaurosis.[1,2] Nevertheless, safety concerns or the availability of suited viral vectors limit their clinical application. Non-viral vectors are a

promising alternative to viral vectors due to a more favourable safety profile, although additional work is required to improve their efficacy.

In this paper, we have shown for the first time the "in vivo" capacity of solid lipid nanoparticles to transfect ocular tissues after administration to the rat eye.[21] In a previous work we had already demonstrated the utility of protamine to promote "in vitro" transfection of ARPE-19 cells with solid lipid nanoparticles.[13] Protamine is a small peptide (Mw 4,000 - 4250) that efficiently condenses DNA. This facilitates the control of formulation processes, prevents the formation of aggregates and contributes to protection against DNase degradation.[22] Moreover, protamine presents NLS sequences with high arginine content and, thus, enhances DNA entry into the nucleus.[22,23] Besides protamine, the present study has also incorporated dextran to the solid lipid nanoparticles. This polynianion biocompatible polysaccharide hampers strong interactions with other components such as serum protein[24] that could have an influence on transfection, especially "in vivo".[25] Furthermore, dextrans are typically used as clathrin-mediated endocytosis markers. Theoretically, the ability of dextran to enhance this cellular uptake by using the endolysosomal pathway[26] needs to be considered since we have previously demonstrated that protamine works most efficiently when clathrins are involved in the entry mechanism of vectors.[13]

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[21] Conley SM, Naash MI. Nanoparticles for retinal gene therapy. Prog Retin Eye Res. 2010;29:376-97.

[22] Ye J, Wang A, Liu C, Chen Z, Zhang N. Anionic solid lipid nanoparticles supported on protamine/DNA complexes. Nanotechnology. 2008;19:285708.

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[25] Finsinger D, Remy JS, Erbacher P, Koch C, Plank C. Protective copolymer for nonviral gene vectors: synthesis, vector characterization and application in gene delivery. Gene Ther. 2000;7:1183-1192

[26] Agarwal A, Gupta U, Asthana A, Jain NK. Dextran conjugated dedritic nanoconstructs as potential vectors for anti-cancer agent. Biomaterials. 2009;30:3588-3596.

The presence of protamine and dextran in the complex modified neither the particle size nor the surface charge of the solid lipid nanoparticles. The complexes we prepared were able to protect the genetic material from DNAses and to release the DNA in the presence of SDS. Since pCEP4-RS1 has a bigger molecular weight (11.1 Kb) than pCMS-EGFP (5.5 Kb), a higher DNA:SLN ratio was necessary for pCEP4-RS1 than for pCMS-EGFP to maintain the protection capacity of the genetic material without affecting the DNA release ability (1:5 vs 1:6 ratios). This optimization is very important for gene therapy applications as a DNA-condensation in excess impedes plasmid release and transfection. In contrast, a low condensation will not protect DNA inside the cell.[16]

Transfection studies with vectors containing pCMS-EGFP plasmid showed that presence of protamine and dextran significantly increased transfection of SLN (Figure 3). The most efficient formulation revealed a dextran-protamine-DNA ratio of 1:2:1. Moreover, the presence of these two components also increased cell viability, regarding control formulation. Our data point to the importance of adequately optimizing the proportion of additives to prepare safe and efficient formulations. It is important to highlight the high transfection level obtained, close to 50% of cells transfected. This is even more intriguing, since ARPE-19 cells are difficult to transfect. For example, Abul-Hassan et al. reported a 25% transfection efficiency for this cell line and other authors achieved even lower transfection rates.[27,28] In previous

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[27] Abul-Hassan K, Walmsley R, Boulton M. Opitimization of non-viral gene transfer to human primary retinal pigment cells. Curr Eye Res. 2000;20:361-366.

[28] Bejjani RA, BenEzra D, Cohen H, Rieger J, Andrieu C, Jeanny JC, et al. Nanoparticles for gene delivery to retinal pigment epithelial cells. Mol Vis. 2005;17:124-132.

studies we achieved transfection levels close to 30% when SLN were combined with protamine,[13] which have been almost doubled in this work by adding dextran to that formulation.

In order to confirm the utility of the vectors for XLRS treatment, we prepared the formulations replacing the model pCMS-EGFP plasmid with a therapeutic plasmid, pCEP4-RS1. Both plasmids were assayed for their transfection capacity in ARPE-19 cells. Retinoschisin, the encoded protein, is an extracellular protein secreted by photoreceptors and bipolar cells that is involved in retinal organization and stability.[15] After transfection, we detected cytoplasmatic retinoschisin by immunochemistry and we also quantified the secreted retinoschisin (Figure 4). Fluorescence microscopy images show that the vectors we prepared (SLN with and without protamine and dextran) were able to produce retinoschisin in the cytoplasm. Moreover, the protein secreted by cells was higher with the formulation containing protamine and dextran. These results are in line with the transfection results of pCMS-EGFP.

While designing and optimizing a vector for gene therapy, the knowledge of internalization mechanisms and intracellular trafficking is very useful, since these limiting steps may be modulated by the formulation. In a previous study carried out by our group,[13] we observed that clathrin mediated endocytosis is needed to achieve transfection when vectors bear protamine. As mentioned above, one of the reasons for the addition of dextran to our vector is the common use of dextrans as clathrin and endolysosomal markers. As expected, the presence of dextrans induced a great internalization via clathrin of dextran-protamine-DNA-SLN vectors that promoted a rise of transfection levels (Figure 6).

Therefore, our previous observations have been confirmed here: indeed, the higher the entry via clathrin, the higher the transfection achieved with vectors bearing protamine is.
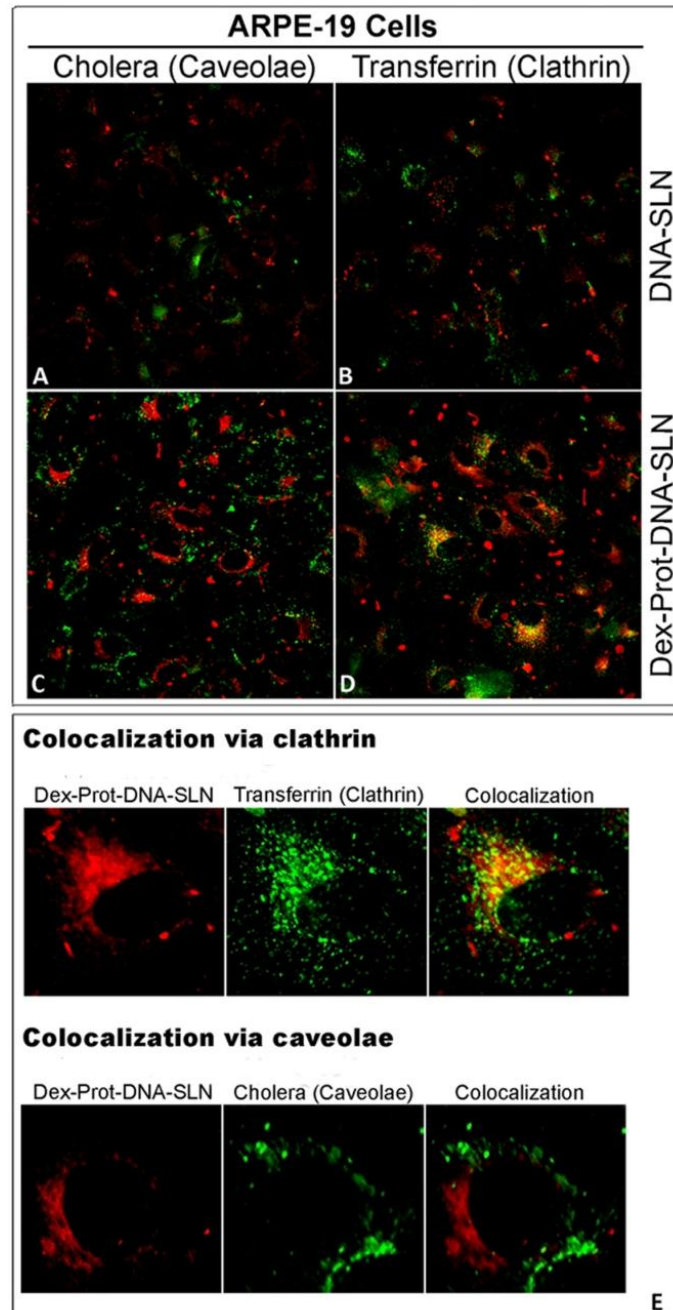

Figure 6. CLSM images of Nile Red-labeled DNA-SLN and dextran-protamine-DNA-SLN vectors in ARPE-19 cells with AlexaFluor488-Cholera toxin (green) at 10 μg/ml (A, C); or with AlexaFluor488-Transferrin (green) at 50 μg/ml (B, D). SLN to DNA ratio (w/w) was 5:1 and dextran-protamine to DNA ratio was 1:2:1.

We carried out a further preliminary "in vivo" study to assess whether the vectors are able to transfect ocular tissues after their administration by different ocular routes: intravitreous, subretinian and topical. In animal models, an "in vitro"-"in vivo" correlation is not necessarily expected due to possible physical barriers.[29,30]

After ocular administration of dextran-protamine-DNA-SLN complexes to the rat eyes, we detected the expression of green fluorescent protein in various types of cells depending on the administration route.

There was a good response in retina ganglion cells when intravitreal injection was employed, but protein expression was poor in RPE cells (Figure 7A). In contrast, after subretinal injection, the vectors were able to transfect RPE cells as well as photoreceptors (Figure 7B).

These results are in line with those obtained in a previous study in which intravitreous administration of adenoviral vectors induced protein expression in the inner retina, whereas RPE cells and outer retina were the main transfected cells after subretinal administration.[31]

This preliminary study may be useful to select the target cells to be transfected depending on the target of gene therapy. Non-viral vectors could be employed to treat different diseases such as glaucoma when the inner retina needs to

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[29] Peeters L, Sanders NN, Braeckmans K, Boussery K, Van de Voorde J, De Smedt SC, et al. Vitreous: a barrier to nonviral ocular gene therapy. Invest Ophthalmol Vis Sci. 2005;46:3553-61.

[30] de la Fuente M, Raviña M, Paolicelli, Sanchez A, Seijo B, Alonso MJ. Chitosan-based nanostructures: a delivery platform for ocular therapeutics. Adv Drug Deliver Rev. 2010;62:100-117.

[31] Colella P, Cotugno G, Auricchio A. Ocular gene therapy: current progress and future prospects. Trends Mol Med. 2009;15:23-31.

be addressed, or retinoschisis and retinitis pigmentosa, if administration reaches the outer retina.[32,33]
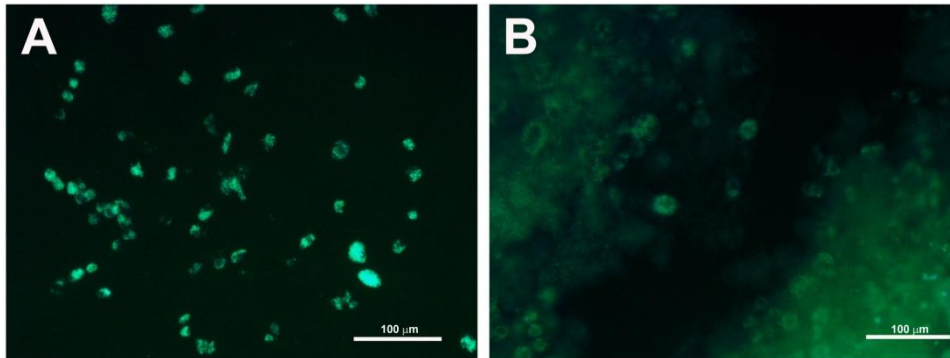
Figure 7. Fluorescence microscopy images of pCMS-EGFP expression in the retina 72h after transfection with the dextran-protamine-DNA-SLN vector. (A) Intravitreal injection showing pCMS-EGFP expression in retinal ganglion cells. (B) Subretinal injection showing expression mainly at the retinal pigment epithelium (RPE). Bar= 100 µm.

Our vectors were also able to transfect corneal cells after topical application (Figure 8). As this administration is a non-invasive route, and cornea immune defense does not induce inflammation, these vectors may be a safe method to treat corneal endothelial dystrophies and to modulate protein production in order to control the corneal microenvironment[34] all this, without the possible limitations that viral vectors present in ocular gene therapy.

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[32] Johnson EC, Guo Y, Cepurna WO, Morrison JC. Neurotrophin roles in retinal ganglion cell survival: Lessons from rat glaucoma models. Exp Eye Res. 2009;88:808-815.

[33] Phelan JK, Bok D. A brief review of retinitis pigmentosa and the identified retinitis pigmentosa genes. Mol Vis. 2000;6:116-124.

[34] Klausner EA, Peer D, Chapman RL, Multack RF, Andurkar SV. Corneal gene therapy. J Control Release. 2007;124:107-133.
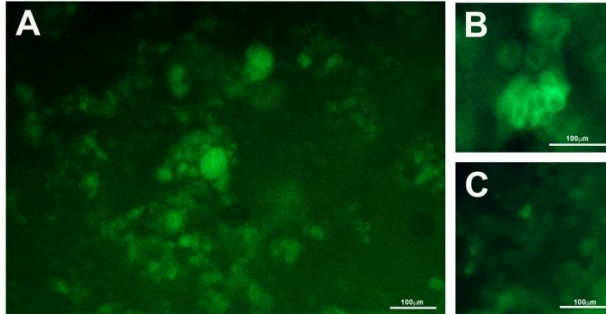
Figure 8. Fluorescence microscopy images of pCMS-EGFP expression in the cornea following topical administration of dextran-protamine-DNA-SLN vector. (A) EFGP expression in the corneal epithelium 72h post-instillation. (B) Detail of rat corneal epithelium. (C) EFGP expression in the corneal epithelium 5 days after the topical administration.

Therefore, we have demonstrated the potential utility of the solid lipid nanoparticles based non-viral vectors loading XLRS1 plasmid. Taking into account that the transfection process depends on the plasmid size, relevant differences of this type of vector loading other therapeutic plasmids with molecular size of the same order than XLRS1 is not expected, although additional studies are necessary. Taken together, we have provided evidence for the potential application of the dextran-protamine-DNA-SLN vectors for the treatment of degenerative retina disorders as well as ocular surface diseases.

5. Acknowledgments

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31. Colella P, Cotugno G, Auricchio A. Ocular gene therapy: current progress and future prospects. Trends Mol Med. 2009;15:23-31.

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DEXTRAN-PROTAMINE-SOLID LIPID NANOPARTICLES AS A NON-VIRAL VECTOR FOR GENE THERAPY: "IN VITRO" CHARACTERIZATION AND "IN VIVO" TRANSFECTION AFTER INTRAVENOUS ADMINISTRATION TO MICE.

Authors

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ABSTRACT
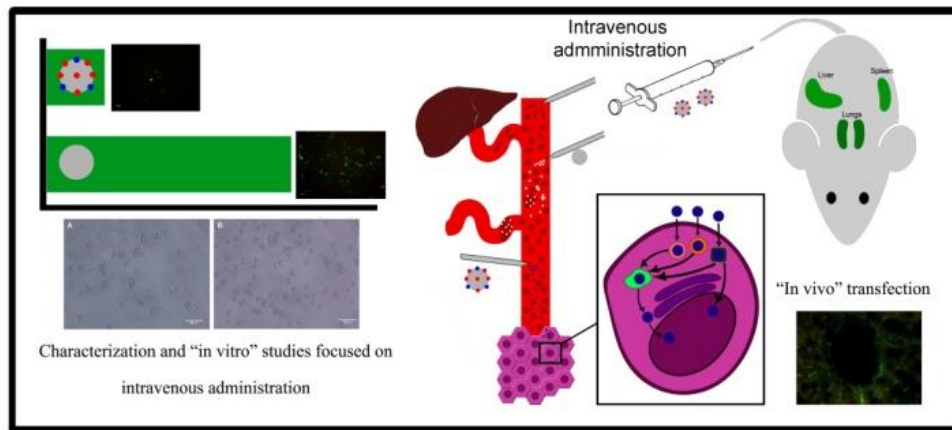
We have evaluated the transfection capacity of a new multicomponent system based on dextran-protamine-solid lipid nanoparticles as a non-viral vector after intravenous administration to mice.

The vector containing the pCMS-EGFP plasmid was characterized in terms of particle size and surface charge. Moreover, the in vitro interaction with erythrocytes, and the transfection capacity, cellular uptake and intracellular trafficking in HEK-293 cells were studied.

The vector showed no agglutination effect, probably due to the presence of dextran. The dextran-protamine-solid lipid nanoparticles were internalized in HEK-293 by caveolae/raft-mediated endocytosis, but they hardly produced transfection. After intravenous administration to BALB/c mice, the expression of EGFP in liver, spleen and lungs was studied. The dextran-protamine-DNA-SLN vector was able to induce

the expression of the green fluorescent protein in the three tissues, and the protein expression was maintained for at least 7 days.

Although additional studies are necessary, this work reveals the promising potential of this new delivery system for the treatment of genetic and non-genetic diseases through gene therapy.

1. INTRODUCTION

Advantages such as safety, low-cost production, high-reproducibility and no limit size of DNA to transport are characteristic features of gene therapy mediated non-viral delivery systems, making it a great alternative to administer intravenously nucleic acid-based therapy.[1] The use of non-viral vectors is limited by their low transfection efficacy, but the search for methods to optimize them is essential to reach the efficacy of gene therapy mediated viral vectors.

A large number of efforts to improve non-viral constructs have been developed following different strategies, and the combinations of several constituents to form hybrid systems have been the most extended and successful approach. The interaction between cationic lipids and DNA, by means of their opposite charges, to form complexes (lipoplexes)[2] stands out as one of the most promising systems. They have been employed in several clinical trials to treat diseases such as cystic fibrosis[3,4] and cancer.[5] Therefore, cationic lipids seems to be the key compound to develop multicomponent carriers.[6]

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In order to enhance the transfection efficacy of these lipids, they can be associated with cell penetratin peptides (CCPs), such as transcriptional activator protein (TAT)[7] and Sweet Arrow Peptide (SAP),[8] which have been used for translocation through the cellular membrane and intracellular gene delivery.[9] This cellular uptake can be carried out through different mechanisms: endocytosis via clathrin, endocytosis via caveolae, clathrin/caveolae-independent endocytosis, etc. The use of constituents that modulate these pathways leads to an improvement in the transfection achieved by gene delivery systems.[10,11] Another strategy consists in facilitating the endolisosomal escape before the degradation of the vector by using polymers such as polyethylenimine or chemicals such as cloroquine, which rupture the endosome when the pH is modified.[12] The nuclear membrane is the last barrier in the transfection process and it can be overcome by means of peptides with nuclear localization signals (NLS). These peptides have the ability to translocate DNA through the nuclear membrane,[13] and they have shown a good response when associated to lipid-based systems.[14]

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Beside those intracellular obstacles, there are specific problems of intravenous administration such as digestion by nucleases and hepatic uptake clearance, which entails a quick elimination of DNA from circulation. Although this drawback is overcome by using gene delivery systems,[15] these present other handicap related to positive charge, which causes aggregation and hemagglutination that may have influence transfection and toxicity. [16, 17] Including anionic polymers such as poly(ethylene glycol) (PEG) diminishes the positive charge of carriers and overcomes the interaction with some components of blood such as erythrocytes.[18]

As a follow-up from previous studies, we have designed new complexes based on previously evaluated solid lipid nanoparticles (SLN) that managed to induce the expression of a foreign protein in organs such as liver and spleen after intravenous administration.[1] In order to improve the results obtained, we have now combined SLN with protamine, a NLS peptide, and dextran, an anionic polysaccharide, which have shown capacity to transfect "in vitro" and successful response after local administration by different routes into rat eye.[19] The objective of this work was to evaluate "in vivo" this new multicomponent system based on dextran-protamine-solid lipid nanoparticles as a non-viral vector after intravenous administration to mice.

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2. MATERIALS AND METHODS

2.1. Materials

Precirol® ATO 5 (Glyceryl palmitostearate) was provided by Gattefossé (Madrid, Spain). Nile Red, protamine sulphate salt from salmon (Grade X), dextran (Mn=3260), and Triton® X-100 were purchased from Sigma-Aldrich (Madrid, Spain). Tween 80 was provided by Vencaser (Bilbao, Spain) and dichloromethane by Panreac (Barcelona, Spain). 1,2-Dioleoyl-3-Trimethylammonium-Propane Chloride Salt (DOTAP) was acquired from Avanti Polar Lipids, Inc.

Plasmid pCMS-EGFP encoding the enhanced green fluorescent protein (EGFP) was purchased from BD Biosciences Clontech (Palo Alto, U.S.) and amplified by Dro Biosystems S.L. (San Sebastián, Spain).

Cell culture reagents were purchased from LGC Promochem (Barcelona, Spain). Antibiotic Normocin™ was acquired from InvivoGen (San Diego, California, US). BD Viaprobe kit was provided by BD Biosciences (Belgium). AlexaFluor488-Cholera toxin and AlexaFluor488-Transferrin were provided by Molecular Probes (Barcelona, Spain), and Fluoromount-G by SouthernBiotech (Coultek, España). Reporter Lysis Buffer was supplied by Promega (USA) and Mirco BCATM Protein Assay Kit by Pierce (USA).

Female Balb/c nude mice weighing 18-22 g (5 weeks of age) were purchased from Harlam Interfauna Ibérica S.L. (Barcelona, Spain).

Primary antibody (polyclonal anti-GFP, IgG fraction) and secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG) were provided by Invitrogen (Barcelona, Spain), and the normal goat serum (NGS) from Chemicon International Inc. (Temecula, CA, USA)

2.2. Production of vectors

The SLN were produced by a solvent emulsification-evaporation technique, previously described by del Pozo-Rodriguez et al.[20] Briefly, Precirol® ATO 5 was dissolved in dichloromethane (5% w/v), and then emulsified in an aqueous phase containing DOTAP (0.4% w/v) and Tween 80 (0.1% w/v). The emulsion was obtained by sonication (Branson Sonifier 250, Danbury) for 30 seconds at 50 W, and after the evaporation of the organic solvent an SLN suspension was formed upon solidification of the Precirol® ATO 5 in the aqueous medium. Finally, the SLN were washed by centrifugation (3,000 rpm, 20 minutes, x 3) using Millipore (Madrid, Spain) Amicon® Ultra centrifugal filters (100,000 MWCO).

The DNA-SLN vector were obtained by mixing the pCMS-EGFP plasmid with an aqueous suspension of SLN under agitation for 30 minutes, which allows the formation of electrostatic interactions between the positive charges of SLN and the negative charges of DNA. The SLN to DNA ratio, expressed as the ratio DOTAP to

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[20] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles: Formulation factors affecting cell transfection capacity. Int J Pharm. 2007;339:261-268.

DNA (w/w), was fixed at 5:1, which has previously demonstrated to fully condense DNA.[20,21]

In order to elaborate the dextran-protamine-DNA-SLN vector, an aqueous solution of dextran was mixed with and aqueous solution of protamine to form the dextran-protamine complexes at w/w ratio of 1:2. Then, pCMS-EGFP plasmid was bound to the dextran-protamine complexes to set dextran-protamine-DNA ratios of 1:2:1 (w/w/w).

Finally, dextran-protamine-DNA complexes were mixed with SLN suspension under agitation for 30 minutes at w/w/w/w ratio of 1:2:1:5. Interaction between free negative charges of DNA and positive charges of SLN led to the formation of the vectors, in which dextran-protamine-DNA complexes are adsorbed on the nanoparticle surface.

2.3. "In vitro" characterization

2.3.1. Size and ζ potential measurements

The size of vectors was determined by photon correlation spectroscopy (PCS), given in volume distribution. ζ potential was measured by Laser Doppler Velocimetry (LDV). Both measurements were performed on a Malvern Zetasizer 3000 (Malvern Instruments, Worcestershire, UK). Samples were diluted in 0.1 mM NaCl (aq.).

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[21] del Pozo-Rodríguez A. Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles for retinal gene therapy: Transfection and intracellular trafficking in RPE cells. Int J Pharm. 2008;360:177-183.

2.3.2. Interaction with erythrocytes

Hemagglutination assay.

A hemagglutination assay was conducted following Kurosaki et al. described previously.[22] Briefly, fresh human blood of 0^+ type was centrifuged at 4000 rpm for 5 min and the plasma and the buffy coat were discarded. Erythrocytes were washed three times by centrifugation at 4000 rpm and were diluted in PBS to a final concentration of 2% (v/v). The naked plasmid, DNA-SLN and dextran-protamine-DNA-SLN vectors were added to erythrocytes suspension at ratio 1:1 (v/v) and incubated for 15 min at room temperature. The sample was placed on a microscope slide and hemagglutination was observed by microscopy.

Hemolysis assay.

A hemolysis assay was conducted following Kurosaki et al. described previously.[22] Briefly, fresh human blood of 0^+ type was centrifuged at 4000 rpm for 5 min and the plasma and the buffy coat were discarded. Erythrocytes were washed three times with PBS by centrifugation at 4000 rpm and were diluted in PBS to a final concentration of 5% (v/v). The naked plasmid, DNA-SLN and dextran-protamine-DNA-SLN vectors were added to erythrocytes suspension at ratio 1:1 (v/v) and incubated for 60 min at room temperature. Then, suspensions were centrifuged at 4000 rpm during 5 min and supernatants were taken to quantify the hemolysis by measuring haemoglobin release at 545 nm in a microplate reader. A lysis buffer was employed for the 100% hemolysis sample.

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[22] Kurosaki T, Kitahara T, Fumoto S, Nishida K, Yamamoto K, Nakagawa H, et al. Chondroitin sulfate capsule system for efficient and secure gene delivery. J Pharm Pharm Sci. 2010;13:351-361.

2.4. "In vitro" transfection

"In vitro" assays were performed with human embryonic kidney (HEK-293) cell line, obtained from the American Type Culture Collection (ATCC). HEK-293 cells were maintained in Eagle's Minimal Essential medium with Earle's BSS and 2 mM L-glutamine (EMEM) supplemented with 10% heat-inactivated horse serum and 1% Normocin™. Cells were incubated at 37ºC with 5% CO_2 in air and subcultured every 2-3 days using trypsin/EDTA. For transfection HEK-293 cells were seeded on 24 well plates at density of 150,000 per well and allowed to adhere overnight.

The formulations were diluted in HBS and added to the cell cultures. In all cases, 2.5 μg of DNA were added. The cells were incubated with the vectors at 37 ºC, and after 4 h, the medium containing the complexes in the wells was refreshed with 1 mL of complete medium. The cells were then allowed to grow for another 72 h. Transfection efficacy was quantified at 12 h, 24 h, 48 h, 72 h and 1 week.

As control, the DNA-SLN vector without protamine whose transfection ability was evaluated in previous works[20] was also assayed.

2.4.1. Flow cytometry mediated analysis of transfection efficacy and cell viability

At 12 h, 24 h, 48 h, 72 h and 1 week, the cells were washed once with 300 μL of PBS, and then detached with 400 μL of 0.05% trypsin-EDTA. The cells were then centrifuged at 1,500 x g, and then resuspended with PBS and directly introduced into a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, California, US). For each sample, 10,000 events were collected.

Transfection efficacy was quantified by measuring the fluorescence of EGFP at 525 nm (FL1). For cell viability measurements, the BD Via-Probe kit was employed. The reagent (5 μL) was added to each sample, and after 10 minutes of incubation, the fluorescence corresponding to dead cells was measured at 650 nm (FL3).

2.4.2. Quantification of expressed green fluorescent protein

At 72 h after vector addition, the cells were washed once with 300 μL of PBS, and then covered with 200 μL of 1X Rerporter Lysis Buffer. The cells were then incubated at room temperature for 15 minutes and scraped. Cell lysates were vortexed during 15 seconds and centrifuged at 12,000 g during 2 minutes at 4ºC. The EGFP of lysates was quantified into a GloMax®-Multi+ Detection System (Promega, USA) with the Blue Optical Kit (510-570 nm) and expressed in relative fluorescence units (RFU). In order to normalize the quantity of EGFP, cellular total protein was measured by using Mirco BCA™ Protein Assay Kit and final results were expressed in RFU/mg protein.

2.5. Cellular uptake of DNA-SLN and dextran-protamine-DNA-SLN vectors

The entry of vectors into the cells was studied quantitatively by flow cytometry. For this purpose SLN were labeled with the fluorescent dye Nile Red (λ = 590 nm) according to a previously reported method.[23] Briefly, Nile Red was

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[23] Borgia SL, Regehly M, Sivaramakrishan R, Mehnert W, Korting HC, Danker K, et al. Lipid nanoparticles for skin penetration enhancement-correlation to drug localization within the particle matrix as determined by fluorescence and parelectric spectroscopy. J Control Release. 2005;110:151-163.

incorporated into the dichloromethane employed to prepare SLN by the emulsification–evaporation technique described above.

Two hours after the addition of vectors, cells were washed three times with PBS and detached from plates. Cells incorporating either labeled vectors were quantified by flow cytometry at 650 nm (FL3). For each sample, 10,000 events were collected.

2.6. Internalization of non-viral vectors

The endocytic processes involved in the internalization of the non-viral systems were analyzed by colocalization studies with AlexaFluor488-Cholera Toxin and AlexaFluor488-Transferrin, which are markers of caveolae mediated endocytosis and clathrin mediated endocytosis, respectively.

Cells were seeded in coverslips and co-incubated for 2 hours with Nile Red labeled vectors and either AlexaFluor488-Cholera toxin (10 μg/ml) or AlexaFluor488-Transferrin (50 μg/ml). Next, the medium was removed and cells were washed with PBS and fixed with paraformaldehyde 4%, which did not interact with the fluorescence of Nile Red or AlexaFluor488. Preparations were mounted on Fluoromount G and, after air-drying, images were obtained with an Olympus Fluoview FV500 confocal microscope using sequential acquisition to avoid overlapping of fluorescent emission spectra. Confocal laser scanning microscopy study (CLSM) images were captured in the General Service of Analytical Microscopy and High Resolution in Biomedicine of the University of the Basque Country (UPV-EHU).

Colocalization results were estimated by means of Manders Overlap Coefficient (R), where 0.6≤R≤1.0 indicates colocalization (overlap of the signals).[24] The value of every coefficient was obtained from 15 images.

2.7. Intravenous administration

Animals were handled in accordance with the Principles of Laboratory Animal Care (http://www.history.nih.gov/laws). Mice were quarantined for approximately 1 week prior to the study. They were housed under standard conditions and had ad libitum access to water and standard laboratory rodent diet.

The dextran-protamine-DNA-SLN vector was injected in standard way into the tail vein in a volume of 100 μL (60 μg of plasmid). Controls were employed by administering free DNA and vectors without plasmid in the same way and volume. The treatment was administered to three mice in each group. Three and 7 days post-injection mice were sacrificed and the liver, lungs and spleen were removed, quick frozen in liquid nitrogen embedded in tissue freezing medium (Jung, Leica) and thin sectioned on a cryostat (Cryocut 3,000, Leica).

2.7.1. Immunolabelling of EGFP in tissue sections

Cryostat sections (7-10 μm) were fixed with 4% paraformaldehyde during 10 min at room temperature. Following washing in PBS, sections were blocked and

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[24] Zinchuk V, Zinchuk O. Quantitive colocalization analysis of confocal fluorescence microscopy images. Curr Protoc Cell Biol. 2008;39:4.19.1–4.19.16.

permeabilized in PBS 0.1M, 0.1% Triton® X-100 and 2% normal goat serum (NGS) for 1 h at room temperature. Then, sections were incubated in primary antibody (polyclonal anti-GFP, IgG fraction) for 2 h at room temperature. Following adequate washing in PBS, sections were incubated in secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG) and DAPI for 45 min at room temperature. Finally, sections were washed again in PBS and coverslipped with Fluoromount G.

Images of the immunolabelled sections were captured with an inverted microscopy equipped with an attachment for fluorescent observation (model EclipseTE2000-S, Nikon).

2.8. Statistical analysis

Results are reported as mean values (SD = standard deviation). Statistical analysis was performed with SPSS 17.0 (SPSS®, Chicago, IL, USA). Normal distribution of samples was assessed by the Shapiro–Wilk test, and homogeneity of variance by the Levene test. The different formulations were compared with ANOVA and student´s t test, whereby differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. "In vitro" characterization

3.1.1. Size and ζ potential measurements

The DNA-SLN vector and the dextran-protamine-DNA-SLN vector showed sizes of 280 nm, and zeta potential about +40 mV (Table 1). The presence of dextran-protamine in the formulations did not result in statistically significant differences in size or zeta potential (p>0.05).

	DNA-SLN	**Dextran-protamine-DNA-SLN**
Size (nm)	283.63 ± 10.68	284.23 ± 11.87
ζ potential (mV)	44.35 ± 6.62	40.60 ± 6.47
Polydispersion Index	0.42 ± 0.03	0.26 ± 0.02

Table 1. Size, ζ potential, polydispersion index and percentage of aggregations of the solid lipid nanoparticles (SLN), the DNA-SLN and the dextran-protamine-DNA-SLN vector.

3.1.2. Interaction with erythrocytes

Agglutination was evaluated incubating the vectors with erythrocytes. Figure 1C shows a light agglutination when erythrocytes were in touch with the DNA-SLN vector, while the use of the dextran-protamine-DNA-SLN vector caused no agglutination (Figure 1D). The same study was carried out with protamine:DNA:SLN vector (w/w/w 2:1:5 ratio) and results were similar to those obtained with the DNA:SLN vector. Figure 2 features the hemolysis activity of erythrocytes after treatment with vectors. As can be seen, hemolysis levels produced by the formulations were similar to levels of the untreated blood, but lower than the positive control (100% hemolysis).
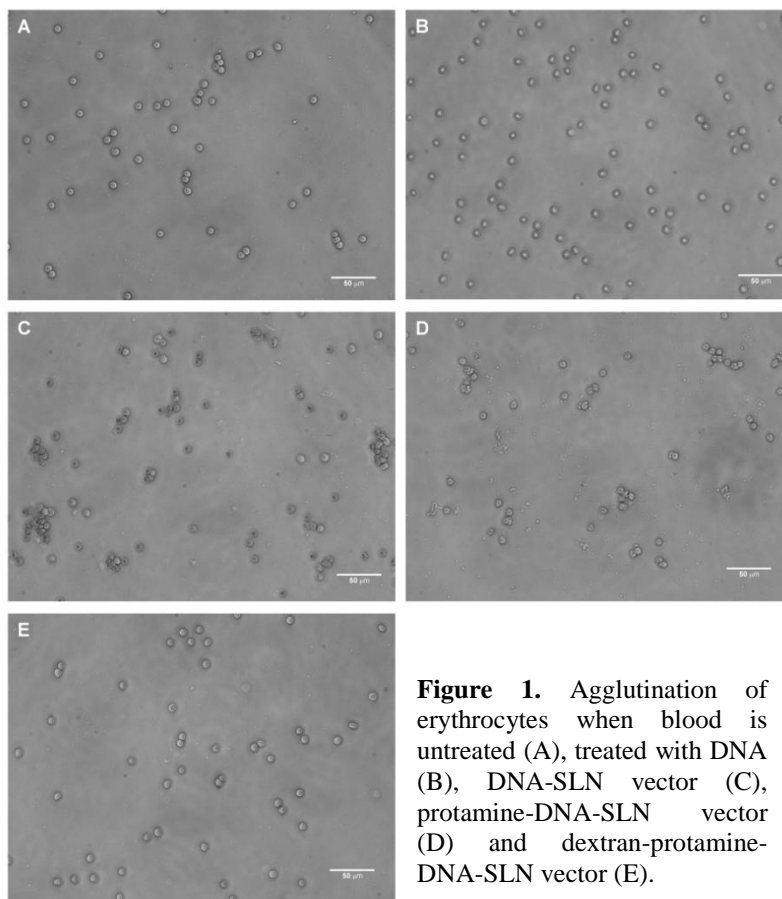
Figure 1. Agglutination of erythrocytes when blood is untreated (A), treated with DNA (B), DNA-SLN vector (C), protamine-DNA-SLN vector (D) and dextran-protamine-DNA-SLN vector (E).
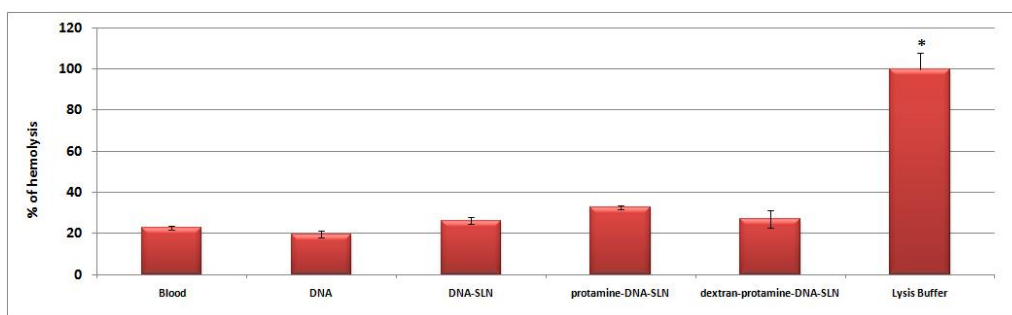
Figure 2. Hemolysis activity of erythrocytes after treatement with the DNA-SLN vector and the dextran-protamine-DNA-SLN vector. Lysis buffer represents 100% hemolysis sample. Error bars represent S.D. (n = 9). * $p < 0.05$.

3.2. "In vitro" transfection and cell viability

DNA-SLN and dextran-protamine-DNA-SLN vectors were tested at different times in HEK-293 cells. The percentage of transfected cells was higher at all times when vector without dextran-protamine complex was used (Figure 3A). The peak of transfection was achieved at 72 hours after adding DNA-SLN vectors (57%), whereas at the same time transfection levels achieved by dextran-protamine-DNA-SLN were only 2.23% of transfected cells ($p<0.01$). Amount of EGFP produced at 72 hours was 8260390 RFU/mg protein with DNA-SLN vector and 348310 RFU/ mg protein with dextran-protamine-DNA-SLN vector (Figure 3B). Cell viability obtained at 72 hours by both formulations was over 80%, significantly higher with dextran-protamine-DNA-SLN vector (95%) ($p<0.01$).
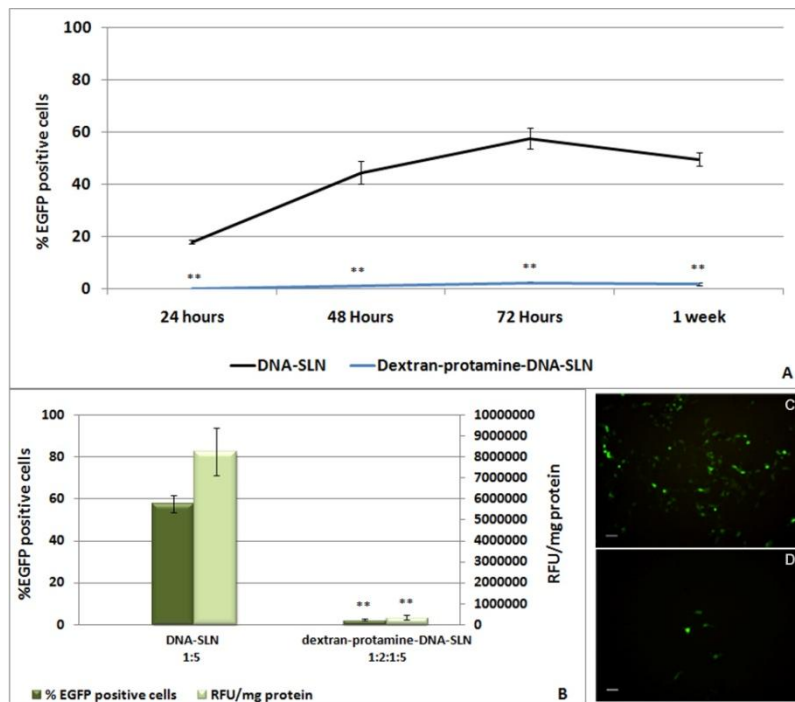
Figure 3. Transfection of vectors in HEK-293 cells over time (A), percentage of EGFP positive HEK-293 cells and RFU/mg protein achieved by vectors at 72h (B), HEK-293 cells transfected by DNA-SLN vector (C) and dextran-protamine-DNA-SLN vector (D).

3.3. Cellular uptake study

The absence of dextran-protamine complex in the formulations caused an increase in the cellular uptake of the DNA-SLN vector with respect to formulation with dextran and protamine. This is shown by a greater rightward shift of the histogram corresponding to the DNA-SLN vector (Figure 4).
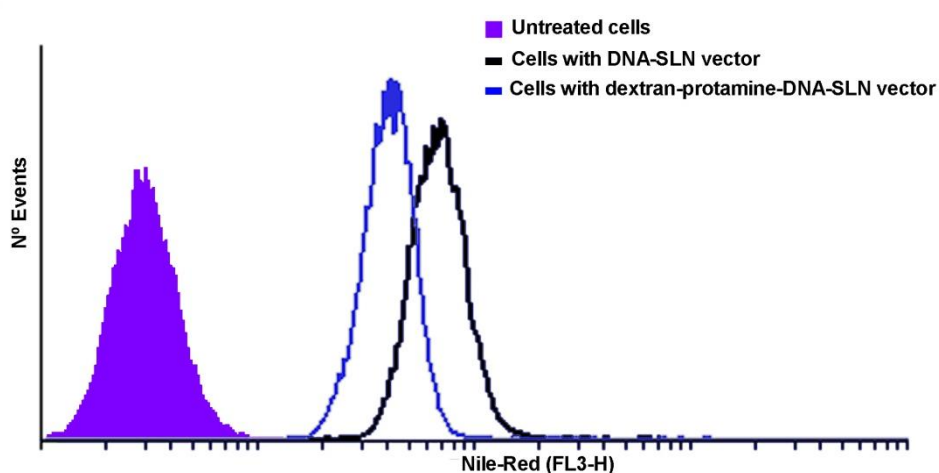
Figure 4. Flow cytometry analysis of cellular uptake study of DNA-SLN vector and dextran-protamine-DNA-SLN vector in HEK-293 cells. Vectors were labeled with Nile Red.

3.4. Internalization of non-viral vectors

After adding endocytosis markers to HEK-293 cells, a higher activity of endocytosis via caveolae (Figure 5A) was observed in comparison with endocytosis mediated via clathrin (Figure 5B).

When HEK-293 cells were treated with our formulations and cholera toxin, DNA-SLN reached a higher colocalization than with the dextran-protamine-DNA-SLN vector. Thus, endocytosis mediated via caveolae is used to a greater extent by

vectors without dextran-protamine (Figure 5). These results were in line with Manders Overlap Coefficients (R), which were 0.676 ± 0.070 with the DNA-SLN vector and 0.426 ± 0.069 with the dextran-protamine-DNA-SLN vector.
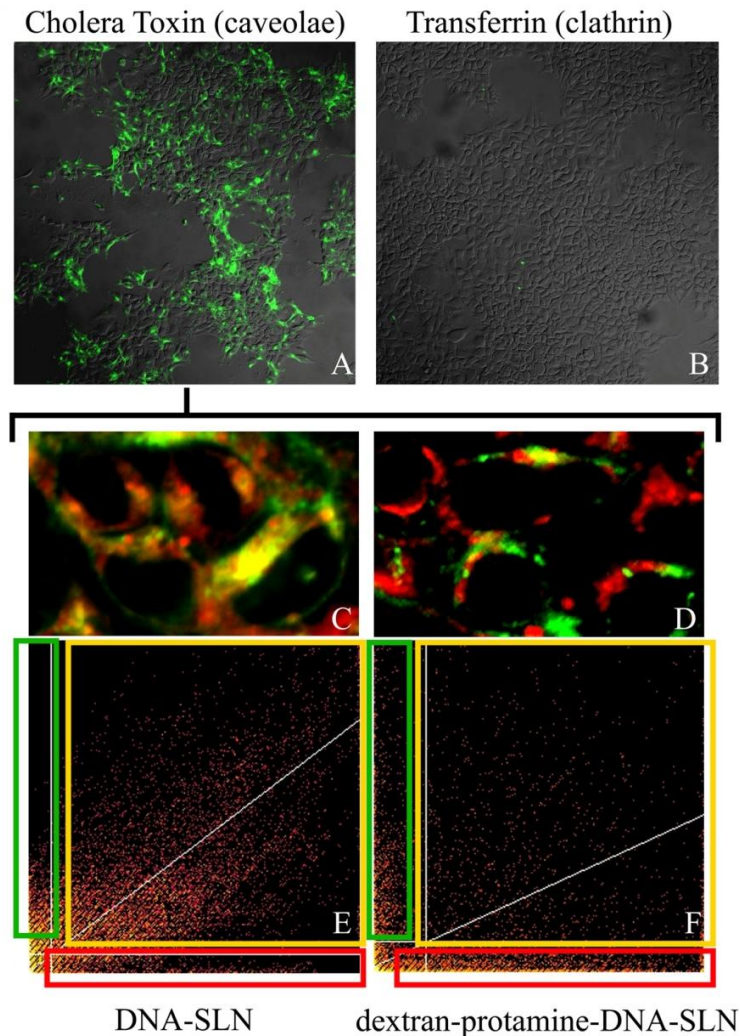
Figure 5. CLSM images (20x) of AlexaFluor 488-Cholera toxin (10 μg/ml) (A) and AlexaFluor 488-Transferrin (50 μg/ml) (B) in HEK-293 cells. Colocalization CLSM images (60x) in HEK-293 cells of AlexaFluor 488-Cholera toxin (10 μg/ml) with Nile Red-labeled DNA-SLN vectors (C) and with dextran-protamine-DNA-SLN vectors (D). SLN to DNA ratio (w/w) was 5:1 and dextran-protamine to DNA ratio was 1:2:1. Colocalization scatterplot of AlexaFluor 488-Cholera toxin with DNA-SLN (E) and with dextran-protamine-DNA-SLN vectors (F); red quadrant: red area; green quadrant: green area; yellow quadrant: colocalization area.

3.5. "In vivo" transfection

Free DNA, and dextran-protamine-DNA-SLN vector were intravenously administered to mice for the "in vivo" assay. In order to ensure that the observed green fluorescence was not an artifact of the immunolabelling, we subjected samples of mice treated with formulations without plasmid to the same procedure with the primary and the secondary antibodies. No green fluorescence was detected. Moreover, no evidence of toxicity was detected.

Mice were sacrificed 3 or 7 days after the intravenous administration. EGFP fluorescence in the tissue sections of mice treated with free DNA was not observed. However, mice treated with the dextran-protamine-DNA-SLN vector show transfection in hepatic, splenic and lung sections.

Twelve (12) sections from each tissue, as a sample of the whole organ were analyzed (Figure 6). At day 3, almost all sections of liver, spleen and lung treated with dextran-protamine-DNA-SLN vector showed EGFP. Transfection remains high at 7 days after intravenous administration in hepatic, spleenic and lung sections. Figure 7 shows transfected sections treated with the dextran-protamine-DNA-SLN vectors.

Figure 6. Percentage of transfected sections of liver, spleen and lung 3 and 7 days after treatment with the dextran-protamine-DNA-SLN vector. The SLN to DNA ratio (w/w) was 5:1 and the dextran-protamine to DNA ratio 1:2:1 (w/w/w).
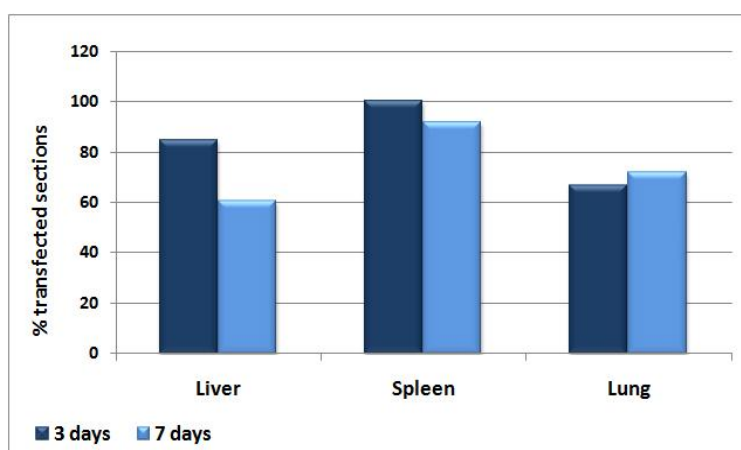
4. DISCUSSION

The successful development of effective non-viral delivery systems is one of the most important goals in the field of gene therapy. Gene delivery efficacy depends on its ability to form stable complexes with nucleic acids, to provide low cytotoxicity, and to disassemble intracellulary to release the nucleic acid.[25] In previous work, we developed solid lipid nanoparticles (SLN) as non-viral vectors to induce protein expression after intravenous administration to mice.[1] In follow-up study, we combined SLN with protamine and dextran. This multicomponent system was able to transfect ocular tissues after local application to rats by different administration routes.[19] In the present study, we have evaluated the transfection capacity of this new vector after systemic administration to mice.

Precondensing the plasmid with protamine contributes to protect it against DNase degradation since this small peptide (Mw 4,000 - 4250) is an efficient DNA condenser. Moreover, protamine presents Nuclear Localization Signals (NLS) with high arginine content that enhances the entry of DNA into the nucleus[26,27] and has previously shown an enhancement of viral and non-viral gene delivery systems,[28] although it has not respond positively when used alone.[29] Dextran is a polyanion

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[25] Gascón AR, Pedraz JL. Cationic lipids as gene transfer agents: a patent review. Expert Opin Ther Pat. 2008;18:515-524.

[26] Sorgi FL, Bhattacharya S, Huang L. Protamine sulfate enhances lipid-mediated gene transfer. Gene Ther. 1997;4:961-968.

[27] Ye J, Wang A, Liu C, Chen Z, Zhang N. Anionic solid lipid nanoparticles supported on protamine/DNA complexes. Nanotechnology. 2008;19:285708.

[28] Lanuti M, Kouri CE, Force S, Chang M, Amin K, Xu K, et al. Use of protamine to augment adenovirus-mediated cancer gene therapy. Gene Ther. 1999;6:1600-1610.

[29] Xu Z, Gu W, Chen L, Gao Y, Zhang Z, Li Y. A smart nanoassembly consisting of acid-labile Vinyl Ether PEG-DOPE and protamine for gene delivery: preparation and "in vitro" transfection. Biomacromolecules 2008;9:3119-3126.

biocompatible polysaccharide that hampers interactions with other components such as serum proteins[30] and could be beneficial for transfection, especially "in vivo".[16]

Vectors were characterized in terms of size and superficial charge. The addition of protamine and dextran to the SLN did not modify the value of these parameters with respect to the DNA-SLN vector (Table 1). Protamine and dextran are positively and negatively charged, respectively, and a compensation of charges may occur. Modifications of particle size depend on the balance between the ability of the peptide to precondense DNA, which would imply a reduction in size, while demanding greater space itself, which would cause an increase in size. Thus, a change in the particle size does not always happen.[8]

Despite lack of significant differences in size and superficial charge, differences in the interaction with erythrocytes between the two formulations were detected (Figure 1). The DNA-SLN vector showed low hemagglutination capacity, whereas dextran-protamine-DNA-SLN vector showed no agglutination. We also detected hemagglutination with a formulation consisting of protamine, DNA and SLN at the 2:1:5 ratio. This indicates that the lack of hemagglutination effect of the dextran-protamine-DNA-SLN is due to the presence of dextran on the nanoparticle surface.

Besides hemagglutination, hemolytic activity, which is also indicative of cytotoxicity, was negligible with all the formulations.

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[30] Maruyama K, Iwasaki F, Takizawa T, Yanagie H, Niidome T, Yamada E, et al. Novel receptor-mediated gene delivery system comprising plasmid/protamine/sugar-containing polyanion ternary complex. Biomaterials. 2004;25:3267-3273.

Regarding "in vitro" transfection capacity, the dextran-protamine-DNA-SLN vector hardly induced transfection in HEK-293 cells. On the contrary, the DNA-SLN vector transfected these cells efficiently (up to 60% of EGFP positive cells 72 hours post-transfection), and transfection lasted for at least 7 days (Figure 3). Transfection studies revealed a good correlation between the percentage of transfected cells, measured by flow cytometry, and the level of EGFP produced, measured by fluorometry.

In order to better understand the lack of transfection of the dextran-protamine-DNA-SLN vector in HEK-293 cells, we studied the uptake and internalization mechanism used by the vector to enter the cells. A significant reduction in the cell uptake of the dextran-protamine-DNA-SLN vector with respect to the DNA-SLN vector was observed (Figure 4), although it is not enough to justify the almost complete reduction of transfection. We also evaluated the entry mechanism of our vectors by means of colocalization studies with caveolae or clathrin-mediated endocytosis markers. Figure 5 depicts that in HEK-293 cells via clathrin is hardly present and, consequently, the two formulations assayed mainly enter by caveolae-mediated endocytosis, with the DNA-SLN vector reaching a higher colocalization with caveolae marker than the dextran-protamine-DNA-SLN vector. As we have already observed in previous works,[11] clathrin-mediated endocytosis is necessary to achieve transfection when protamine is added to lipid nanoparticles. The low activity of this uptake mechanism in HEK-293 cells compared to endocytosis via caveolae may justify the scarce transfection attained by the dextran-protamine-DNA-SLN vector.

In spite of the low transfection observed in HEK-293 cells, after the intravenous administration to mice, lipid nanoparticles with protamine and dextran managed to obtain a significant response in liver, spleen and lung. Three days after the administration of the formulation the percentage of positive sections (presence of EGFP) was 70% in lung, 85% in liver and 100% in spleen. Transfection was still detected in the three organs 7 days post-administration, and it even increased in lung. The formulation DNA-SLN, previously assayed in mice by intravenous route,[1] also showed transfection in liver and spleen but not in lung. Moreover, protein expression decreased greatly 7 days after administration.
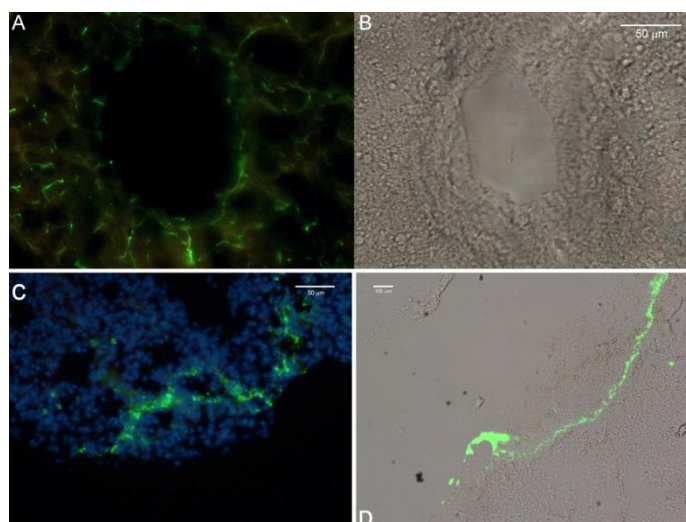
Figure 7. Fluorescence microscopy images of transfected sections of liver at 60x (A-B), lung at 40x (C) and spleen at 10x (D) after the immunolabelling of EGFP (green). Cell nucleuses were labeled with DAPI (blue) in C.

The increase of transfection over time by the dextran-protamine-DNA-SLN vector with respect to the DNA-SLN vector suggests a longer circulation time.[31,32] It is well known that positive charges of cationic complexes may interact with other lipid particles, serum proteins and erythrocytes, leading to aggregation in the blood

[31] Chrastina A, Kerri AM, Schnitzer JE. Overcoming *"in vivo"* barriers to targeted nanodelivery. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2011;3:421-437.

[32] Fischer D, Osburg B, Pertersen H, Kissel T, Bickel U. Effect of poly(ethylene imine) molecular weight and pegylation on organ distribution and pharmacokinetics of polyplexes with oligodeoxynucleotides in mice. Drug Metab Dispos. 2004;32:983-992.

stream and hemagglutination[17] and, consequently, rapidly cleared from the circulation. The "in vitro" study revealed that dextran was able to avoid the erythrocytes aggregation and therefore, the longer circulation stay in the bloodstream can be due to the presence of dextran on the nanoparticle surface. The high DNA condensation of protamine that enhances the nuclease resistance may also result in an extended stay of plasmid in the organism. This fact could be also involved in the transfection obtained in lung. Moreover, the presence of NLS in protamine, which improves the nuclear envelope translocation and its capacity to facilitate transcription,[33] may also improve the transfection efficacy "in vivo". The anatomical and physiological characteristics of liver and spleen, more specifically the discontinuous endothelium, facilitate the uptake of the nanoparticles. This contributes to efficiently transfect these organs. These results suggest the potential usefulness of this vector when liver and spleen are the target organs,[31] such us, for example, in the treatment of diseases such as liver or spleen tumors,[34] hepatitis B[35] or for the design of DNA vaccines.[36] Liver could also be used as a depot organ to produce large amounts of a therapeutic enzyme that is secreted to the bloodstream and recaptured by other organs.[37] The ability of our new vector to transfect in lung makes it useful as a potential system for the treatment of diseases such as acute respiratory distress syndrome (ARDS), cancer, asthma,

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[33] Masuda T, Akita H, Harashima H. Evaluation of nuclear transfer and transcription of plasmid DNA condensed with protamine by microinjection: The use of a nuclear transfer score. FEBS Lett. 2005;579:2143-2148.

[34] Tang H, Tang XY, Liu M, Li X. Targeting alpha-fetoprotein represses the proliferation of hepatoma cells via regulation of the cell cycle. Clin Chim Acta. 2008;394:81-88.

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[36] Raska M, Moldoveanu Z, Novak J, Hel Z, Novak L, Bozja J, et al. Delivery of DNA HIV-1 vaccine to the liver induces high and long-lasting humoral immune responses. Vaccine. 2008;26:1541-1551.

[37] Mango RL, Xu L, Sands MS, Vogler C, Seiler G, Schwarz T, et al. Neonatal retroviral vector-mediated hepatic gene therapy reduces bone, joint, and cartilage disease in mucopolysaccharidosis VII mice and dogs. Mol Genet Metab. 2004;82:4-19.

emphysema and cystic fibrosis (CF).[38] Although these are clearly promising results, additional studies would be needed to assess the real potential of this new vector.

In recent years, the development of carriers to target caveolae-mediated endocytosis is emerging as a strategy to improve non-viral vectors.[31,39,40] We have developed a new gene delivery system that, when internalized by caveolae/raft-mediated endocytosis, was not able to transfect "in vitro", but managed a better response "in vivo" than the DNA-SLN vector, which was also internalized by caveolae/raft-mediated endocytosis and transfected efficiently "in vitro". This lack of "in vitro"-"in vivo" correlation, which is accepted for this kind of formulations, jointly with the lack of adequate methods to better understand the process of endocytosis and intracellular trafficking of gene carriers "in vivo",[40] justifies the inclusion of studies with animal models during early stages of the development of gene delivery systems. In summary, we have developed a non viral vector based on dextran-protamine-DNA-SLN able to induce the expression of a foreign protein (green fluorescent protein) in the spleen, liver and lung after intravenous administration to mice, and expression that was maintained for at least 7 days. Although additional studies are necessary, this work reveals the promising potential of this new delivery system for the treatment of genetic and non-genetic diseases through gene therapy.

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[39] Liu C, Yu W, Chen Z, Zhang J, Zhang N. Enhanced gene trasnsfection efficiency in CD13-positive vascular endothelial cells with targeted poly (lactic acid) –poly (ethylene glycol) nanoparticles through caveolae-mediated endocytosis. J Control Release. 2011;151:162-175.

[40] Sahay G, Alakhova DY, Kavanov AV. Endocytosis of nanomedicines. J Control Release. 2010;145:182-195.

5. Acknowledgements

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20. del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR, Pedraz JL, Solid lipid nanoparticles: Formulation factors affecting cell transfection capacity. Int J Pharm. 2007;339:261-268.

21. del Pozo-Rodríguez A. Delgado D, Solinís MA, Gascón AR, Pedraz JL. Solid lipid nanoparticles for retinal gene therapy: Transfection and intracellular trafficking in RPE cells. Int J Pharm. 2008;360:177-183.

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33. Masuda T, Akita H, Harashima H. Evaluation of nuclear transfer and transcription of plasmid DNA condensed with protamine by microinjection: The use of a nuclear transfer score. FEBS Lett. 2005;579:2143-2148.

34. Tang H, Tang XY, Liu M, Li X. Targeting alpha-fetoprotein represses the proliferation of hepatoma cells via regulation of the cell cycle. Clin Chim Acta. 2008;394:81-88.

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40. Sahay G, Alakhova DY, Kavanov AV. Endocytosis of nanomedicines. J Control Release 2010;145:182-195.

Gene therapy, which involves systems for the targeted delivery of therapeutic genetic material into cells, has a great potential for treating a large number of pathologies such as cancer,[1,2] ocular,[3] skin[4] or neurodegenerative diseases.[5] Non-viral vectors are safer, cheaper, and more reproducible than viral vectors, and have no limitation in the size of DNA they can transport, but their transfection efficacy is still low.[6] Therefore, the enhancement of non-viral vectors is a priority in the field of gene therapy, in order to manage effective and safe vectors.

A large number of efforts to improve non-viral constructs has been developed following different strategies, and combination of several constituents to form hybrid systems has become the most extended and successful approach. The interaction between cationic lipids and DNA, by means of their opposite charges to form complexes (lipoplexes),[7] stands out as the most promising system, and is the key compound to develop multicomponent carriers.[8] In order to enhance the transfection efficacy of these lipids, they can be associated with cell penetratin peptides (CCPs), which have been used for translocation through the cellular membrane and intracellular gene delivery.[9] This cellular uptake can be carried out through different mechanisms: endocytosis via clathrin, endocytosis via caveolae, clathrin/caveolae-

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[1] Khalighinejad N, Hairi H, Behnmfar O, Yousefi A, Momeni A. Adenoviral gene therapy in gastric cancer: A review. World J Gastroenterol. 2008;14:180-184.

[2] Takakashi S, Ito Y, Hatake K, Sugimoto Y. Gene therapy of breast cancer. – Review of clinical gene therapy trials for breast cancer and MDR1 Gene Therapy Trial in Cancer Institute Hospital. Breast Cancer. 2006;13:8-15.

[3] Chung DC, Lee V, Maguire AM. Recent advances in ocular gene therapy. Curr Opin Ophtalmol. 2009;20:377-381.

[4] Pfützner W. Vectors for gene therapy of skin diseases. JDDG. 2010;8:582-590.

[5] Ralph GS, Binley K, Wong LF, Azzouz M, Mazarakis ND. Gene therapy for neurodegenerative and ocular diseases using lentiviral vectors. Clin Sci (Lond). 2006;110:37-46.

[6] del Pozo-Rodríguez A, Pujals S, Delgado D, Solinís MA, Gascón AR, Giralt E, et al. A proline-rich peptide improves cell transfection of solid lipid nanoparticles-based non viral vectors. J Control Release. 2009;133:52-59.

[7] Ma B, Zhang S, Jiang H, Zhao B, Lv H. Lipoplex morphologies and their influences on transfection efficiency in gene delivery. J Control Release. 2007;123:184-194.

[8] Koyanova R, Tenchov B. Recent patents in cationic lipid carriers for delivery of nucleic acids. Recent Pat DNA Gene Seq. 2011;5:8-27.

[9] Gupta B, Levchenko TS, Torchilin VP. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. Adv Drug Deliv Rev. 2005;57:637-651.

independent endocytosis, etc. The use of constituents that modulate these pathways leads to improve transfection achieved by gene delivery systems.[10] Another strategy consists in facilitating the endolisosomal escape before vector degradation by using polymers such as polyethylenimine or chemicals such as cloroquine, which rupture the endosome when the pH is modified.[11] The nuclear membrane is the last barrier in the transfection process and it can be overcome by means of peptides with nuclear localization signals (NLS), which have the ability to translocate DNA through the nuclear membrane.[12]

In this work a non-viral gene delivery system has been developed, based on solid lipid nanoparticles (SLN) in combination with protamine and dextran. Protamine is an USP (United States Pharmacopeia) compound isolated from the sperm of mature fish. This peptide condenses DNA and presents nuclear localization signal (NLS) with high presence of arginine residues (Box 1), which makes this peptide able to translocate molecules such as DNA from the cytoplasm to the nucleus of living cells.[13,14] On the other hand, dextran is a polynianion biocompatible polysaccharide that hampers strong interactions with other components such as serum proteins[15] that could influence transfection, especially "in vivo".[16]

[10] Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae- medaited endocytosis in gene transfer mediated by lipo- and polyplexes. Mol Ther. 2005;12:468-474.

[11] Varkouhi AK, Scholte M, Storm G, Haisma HJ. Endosomal escape pathways for delivery of biologicals. J Control Release. 2010;151:220-228.

[12] Zanta MA, Belguise-Valledier P, Behr JP. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleous. Proc Natl Acad Sci USA. 1999;96:91-96.

[13] Balhorn R. The protamine family of sperm nuclear proteins. Genome Biol. 2007;8:227.

[14] Biegeleisen K. The probable structure of the protamine-DNA complex. J Theor Biol. 2006;241:533-540.

[15] Maruyama K, Iwasaki F, Takizawa T, Yanagie H, Niideome T, Yamada E, et al. Novel receptor-mediated gene delivery system comprising plasmid/protamine/sugar-containing polyanion ternary complex. Biomaterials. 2004;25:3267-3273.

[16] Finsinger D, Remy JS, Erbacher P, Koch C, Plank C. Protective copolymer for nonviral gene vectors: synthesis, vector characterization and application in gene delivery. Gene Ther. 2000;7:1183-1192.

Aminoacids sequence of protamine and its high content in arginine (A). DNA condensation by protamine (B).[13] Dextran structure (C).
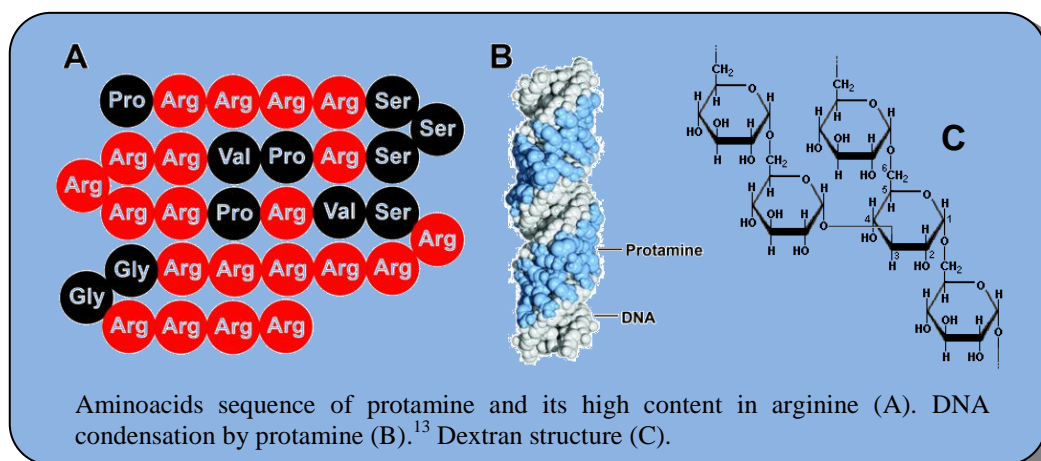
Box 1. *Protamine and dextran.*

The particle size and zeta-potential of a formulation containing SLN, DNA, protamine and dextran at different ratios were measured. Their ability to protect the DNA against DNAse and to release it from vectors was also studied, since this balance is essential in the transfection process.[17] Moreover, vectors were tested "in vitro" in order to study their cellular uptake, intracellular and nuclear trafficking, and transfection efficacy by using the plasmid that codifies the enhanced green fluorescent protein (EGFP).

These assays were conducted in different cell lines since it is well-known that transfection efficiency of nanoparticular vectors is conditioned by cell line-dependent factors such as division rate, internalization pathway and intracellular trafficking.[18,19]

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[17] del Pozo-Rodríguez A, Delgado D, Solinís MA, Gascón AR. Pedraz JL. Solid lipid nanoparticles: Formulation factors affecting cell transfection capacity. Int J Pharm. 2007;339:261-268.

[18] Douglas KL, Piccirillo CA, Tabrizian M. Cell line-dependent internalization pathways and intracelular trafficking determine transfection efficiency of nanoparticles vectos.Eur J Pharm Biopharm. 2008;68:676-687.

[19] Duan Y, Zhang S, Wang B, Yang B, Zhi D. The biological routes of gene delivery mediated by lipid based non-viral vectors. Expert Opin Drug Del. 2009.12;1351-1361.

Therefore, a human embryonic kidney (HEK-293) cell line and a human retinal pigmented epithelial (ARPE-19) cell line were used in these experiments: HEK-293 cells (Figure 1A) due to their widespread use in transfection studies, and ARPE-19 cells (Figure 1B) because of their utilization as a cell line model of retinal diseases.[20]

Moreover, these retinal pigment epithelial cells were transfected with a plasmid that codifies retinoschisin, whose absence causes X linked juvenile retinoschisis (XLRS).[21]
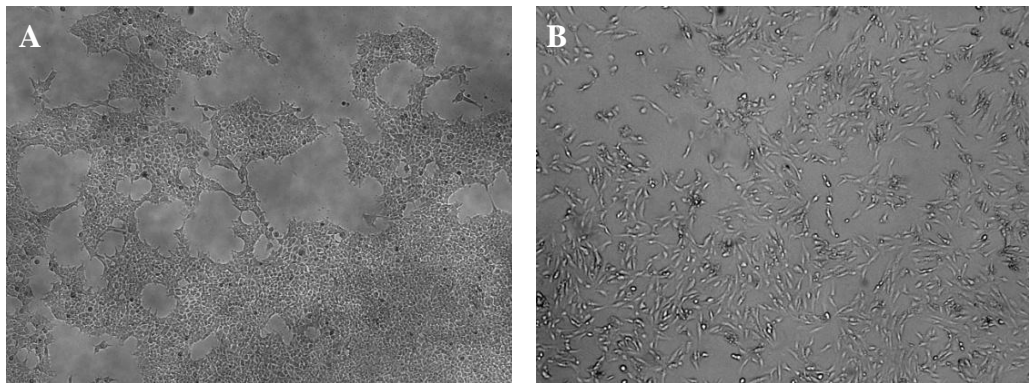
Figure 1. *HEK-293 cells (A) and ARPE-19 cells (B).*

Nevertheless, in order to complete an apprpiate development and evaluation of gene delivery systems, studies in an animal model are necessary since the "in vitro"-"in vivo" correlation is difficult because of the biological and physiological barriers the systems need to overcome in a living animal, namely, circulation within

[20] Bejjani RA, Benzra D, Cohen H, Rieger J, Andrieu C, Jeanny JC. Nanoparticles for gene delivery to retinal pigment epitelial cells. Mol Vis. 2005;17:124-132.
[21] Sergeev YV, Caruso RC, Meltzer MR, Smaoui, MacDonald IM, Sieving PA. Molecular modeling of retinoschisin with functional analysis of pathogenic mutations from human X-linked retinoschisis. Hum Mol Genet. 2010;19:1302-1313.

the bloodstream, tissue distribution, cell uptake and intracellular trafficking.[22] Thus, our formulations were tested by local administration as well as systemic administration in order to prove their capacity to transfect "in vivo". Firstly, vectors were administrated into Wistar rat eyes by using different administration routes to study the application of our formulations as a tool for the treatment of hereditary and acquired ocular disorders. The inherited retinal degenerations have an estimated prevalence of 1:4,000 with a progressive and often untreatable course,[23] and the clinical potential of gene therapy has been recently demonstrated by major improvements of visual function in first clinical trials with patients suffering from Leber`s congenital amaurosis.[24] Subsequently, an intravenous administration through tail vein in Balb/C mice was conducted. Thus, our vectors could widen their targets and treat genetic autosomal dominant or recessive single gene disorders such as hemophilia, many forms of cancer, infectious diseases like HIV, inflammatory conditions and intractable pain.[25]

1. INCORPORATION OF PROTAMINE INTO THE DNA-SLN VECTOR

As mentioned above, protamine is an NLS peptide used as a transfection enhancer due to its ability to condense DNA and translocate it into the nucleus;[26] moreover protamine improves intra-nuclear transfection.[27] Initially, DNA was

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[22]del Pozo-Rodríguez A, Delgado D, Solinís MA, Pedraz JL, Echevarria E, Rodríguez JM, et al. Solid lipid nanoparticles as potential tools for gene therapy: in vivo protein expression after intravenous administration. Int J Pharm. 2010;385:157-162.

[23] Hamel C. Retinitis pigmentosa. Orphanet J Rare Dis. 2006;1:40.

[24] Maguire AM, High KA, Auricchio A, Wright JF, Pierce EA, Testa F, et al. Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. Lancet. 2009;374:1597-1605.

[25] Flotte TR. Gene therapy: the first two decades and the current state-of-the-art. J Cell Physiol. 2007;213:301-305.

[26] Liu J, Guo S, Li Z, Gu J. Synthesis and characterization of stearyl protamine and investigation of their complex with DNA for gene delivery. Colloids Surf B Biointerfaces. 2009;73;36-41.

[27] Masuda T, Akita H, Harashima H. Evaluation of nuclear transfer and transcription of plasmid DNA condensed with protamine by microinjection: The use of nuclear transfer score. FEBS Lett. 2005;579:2143-2148.

precondensed at different protamine:DNA ratios (0.5:1, 1:1, 2:1, 3:1 and 5:1) and then, protamine-DNA complexes were adsorbed in SLN. The SLN to DNA ratio, expressed as the ratio of DNA to DOTAP (w/w), was fixed at 1:5. SLN were previously formed by emulsification/evaporation technique[17] (Box 2).
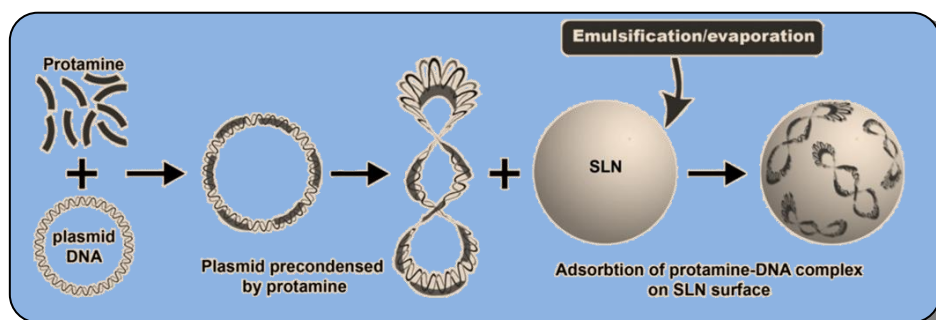
Box 2. *Preparation of protamine-DNA-SLN vector.*

As expected, the capacity of protamine to condense DNA depended on the protamine to DNA ratio. Box 3 shows that a ratio of at least 2:1 was needed to bind all DNA. It can be observed that when a protamine:DNA ratio at 2:1 was used to form the protamine-DNA-SLN vector, the DNA protection capacity increased with respect to DNA-SLN complexes. Another important issue to be considered is the ability of the nanoparticles to deliver the DNA; the higher the amount of protamine in the vector, the more difficult the release of plasmid was. However, the formulation prepared at a protamine:DNA:SLN ratio of 2:1:5 possessed the suitable characteristics to transfect because this formulation increased the protection capacity of the DNA-SLN vector without hampering plasmid release. Furthermore, the inclusion of protamine modified neither particle size of the vector nor its zeta potential, and all formulations prepared had a particle size around 250 nm (S.D. = 43.20) and a zeta potential about +35 mV (S.D. = 2.40). Modifications of particle size depend on the balance between the ability of the peptide to precondense DNA, which would imply a reduction in size, while demanding greater space itself, which would cause an increase in size. Thus, a change in particle size does not always happen.[6]
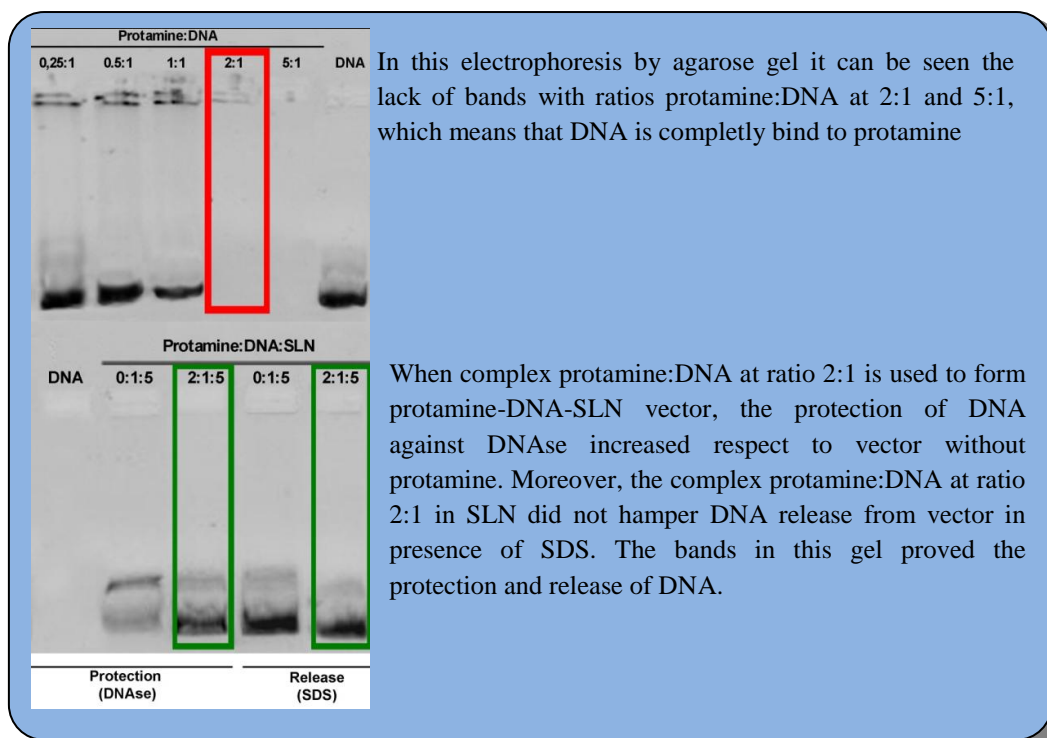
Box 3. *Influence of protamine in protection and release of DNA.*

The good features of the protamine-DNA-SLN vector at ratio 2:1:5 were evident when ARPE-19 cells were transfected with this formulation as the most effective since it reached the highest transfection levels (29%) and improved six fold the levels achieved by the DNA-SLN vector (p<0.05). Efficacy decreased when higher and lower ratios of protamine were used in the formulation because of the difficulty to release the plasmid and the consumption of NLS sequences, respectively.[27] Unexpectedly, the incorporation of protamine to our vector decreased the transfection in HEK-293 cells (from 60% to 2% transfected cells) in a protamine concentration manner (p<0.05). Cell vitality increased when vectors with protamine were used in both cell lines (p<0.05), although it always remained over 80%.

Transfection levels in ARPE-19 cells

Protamine:DNA:SLN	Subset for alpha = 0.05 (Student-Newman-Keuls)			
	1	2	3	4
0:1:5	4.283			
0.5:1:5	4.773			
1:1:5	5.437			
2:1:5				28.880
3:1:5			11.807	
5:1:5		7.300		
% EGFP positive cells				

Transfection levels in HEK-293 cells

Protamine:DNA:SLN	Subset for alpha = 0.05 (Student-Newman-Keuls)			
	1	2	3	4
0:1:5				62.576
0.5:1:5			42.183	
1:1:5		24.693		
2:1:5	1.798			
3:1:5	1.990			
% EGFP positive cells				

EGFP in ARPE-19 cells (4x)

DNA-SLN (1:5) — Protamine-DNA-SLN (2:1:5)

The formulations are classified depending on the statistically significant differences of their transfection levels (at $p < 0.05$).
In ARPE-19 cells (above) the best result was achieved by protamine-DNA-SLN vector (2:1:5), while in HEK-293 cells (below) with DNA-SLN vector without protamine (0:1:5).

Box 4. *Influence of protamine in transfection levels.*

As the passage of DNA into the nucleus is promoted by mitotic activity,[28] the low-division rate of ARPE-19 cells makes the nuclear entry of DNA difficult. The NLS of protamine would favor this fact and improve transfection efficacy. In contrast, entry of DNA into the nucleus should not be a limiting step in transfection of HEK-293 cells because of its high-division rate, and inclusion of protamine in the vector might not affect or increase transfection in these cells. However, the significant transfection levels drop in these cells when protamine was included suggests that this peptide may also affect other critical limiting steps in the transfection process such as cellular uptake and intracellular trafficking.

The cellular uptake study after 24 h showed that the presence of protamine in the formulations did not modify the percentage of ARPE-19 cells that captured

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[28] Mortimer I, Tam P, MacLachlan I, Graham RW, Saravolac EG, Joshi PB. Cationic lipid-mediated transfection of cells in cultura requires mitotic activity. Gene Ther. 1999;6:403-411.

vectors (77% for DNA-SLN vs. 72% for protamine-DNA-SLN at ratio 2:1:5). Moreover, there was a decrease of cell uptake in HEK-293 cells, from 75% to 45% when the vector with protamine was used. As in ARPE-19 cells, no correlation between uptake and transfection was found, since a decrease of 30% in cell uptake caused an almost complete inhibition of transfection. This lack of correlation between cell internalization and transfection has been previously described by other authors.[29]

Regarding intracellular trafficking, two of the best known endocytosis mechanisms were studied: clathrin-mediated endocytosis, which entails lysosomal activity, and caveolae-mediated endocytosis, which avoids the lysosomal pathway (Box 5).[30]

Endocytosis has been postulated as the main entry mechanism for non-viral systems. Various endocytosis mechanisms have been described to date: phagocytosis, pinocytosis, endocytosis via clathrin, endocytosis via caveolae, clathrin/caveolae-independent endocytosis, etc. Clathrin-mediated endocytosis (left) leads to an intracellular pathway in which endosomes fuse with lysosomes, which degrade their content, whereas caveolae/raft mediated endocytosis (right) avoids the lysosomal pathway and its consequent vector degradation.

(Images modified from Sahay et al. 2010.[30])

Box 5. *Endocytosis mechanism*

[29] Mannermaa E, Rönkkö S, Ruponen M, Reinisalo M, Urtti A. Long-lasting secretion of transgene product from differentiated and filter-grown retinal pigment epithelial cells after non viral gene transfer. Curr Eye Res 2005;30:345-353.
[30] Sahay G, Alakhova DY, Kavanov AV. Endocytosis of nanomedicines. J Control Release. 2010;145:182-195.

Caveolae-mediated endocytosis was labeled with AlexaFluor488-Cholera Toxin and clathrin-mediated endocytosis with AlexaFluor488-Transferrin. Using flow cytometry and Confocal Laser Scanning Microscopy (CLSM) we observed that whereas in ARPE-19 cells both mechanisms are presented in a similar way, in HEK-293 cells endocytosis via caveolae is much more active than endocytosis via clathrin (Figure 2).
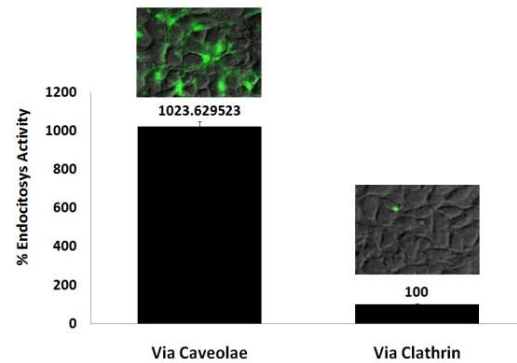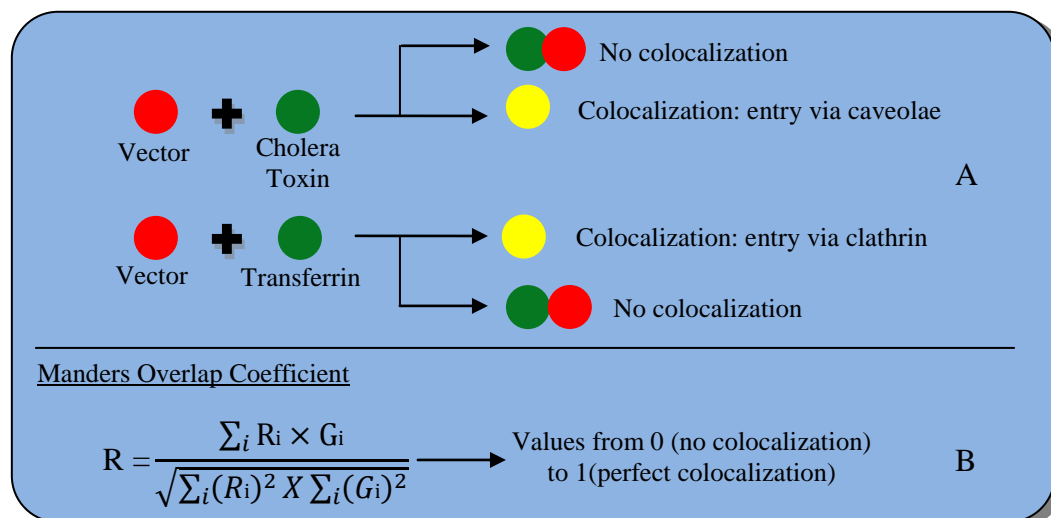

Figure 2. *Endocytosis activity in HEK-293 cells. Clathrin-mediated endocytosis activity was set to 100%.*

Colocalization study was carried out by means of CLSM after labeling SLN with Nile Red in order to colocalize them with markers of endocytosis. Then, results of images were estimated by means of Manders Overlap Coefficient (R) (Box 6).[31,32]

Manders Overlap Coefficient

$$R = \frac{\sum_i R_i \times G_i}{\sqrt{\sum_i (R_i)^2 \, X \, \sum_i (G_i)^2}}$$

Values from 0 (no colocalization) to 1(perfect colocalization)

Box 6. *Colocalization schema (A) and Manders Coefficient (B)*

[31] Zinchuk V, Zinchuk O. Quantitative colocalization analysis of confocal fluorescence microscopy images. Curr Protoc Cell Biol. 2008;39:4.19.1-4.19.16.

[32] Manders EMM, Verbeek FJ, Aten JA. Measurement of co-localization of objects in dual-colour confocal images. J Microsc. 1993;169:375-382.

Images of the CLSM study showed that the protamine-containing vector presented a higher colocalization with transferrin in ARPE-19 cells (R = 0.641 ± 0.039 for protamine-DNA-SLN vector vs. R = 0.424 ± 0.124 for DNA-SLN vector) (Figure 3), which indicates that protamine induced a shift in the internalization mechanism from caveolae/raft to clathrin endocytosis. Moreover, in both cell lines DNA-SLN vector used mainly endocytosis via caveolae route. These results, taken in conjunction with the low activity of endocytosis via clathrin in HEK-293 cells, suggest that the lysosomal activity of this mechanism is needed to the release protamine-DNA complex from SLN and in that way it would be able to enter the nucleus.
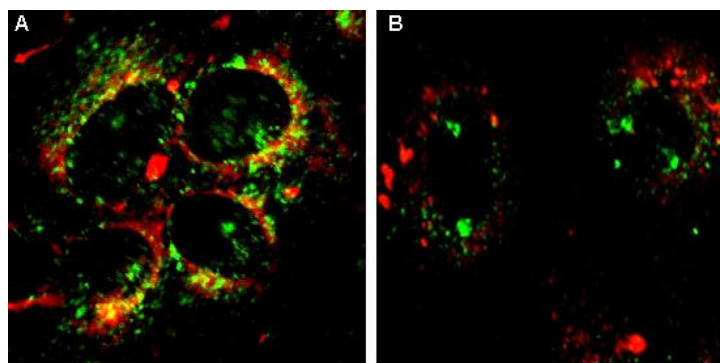

Figure 3. *Endocytosis via clathrin in ARPE-19 cells.* Colocalization of transferrin (green) with vector with protamine (A) and without protamine (B) (red).

Since the internalization pathway conditions later steps, we investigated the intracellular disposition of vectors prepared with EMA-labelled DNA. We observed that DNA was highly condensed and near the nucleus after the treatment of both cell lines with the formulation containing protamine. However, when ARPE-19 cells were treated with DNA-SLN (without protamine), the plasmid was broadly distributed in the cytoplasm (Figure 4). This fact proves that nuclear localization signals of protamine help DNA to reach and enter into the nucleus, which is necessary to transfect cells with slow division rate such as ARPE-19 cells.[33]

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[33] Boulanger C, Di Giorgio C, Vierling P. Syntehsis of acridine-nuclear localization signal (NLS) conjugates and evaluation of their impacto n lipoplez and polyples-based transfection. Eur J Med Chem. 2005;40:1295-1306.
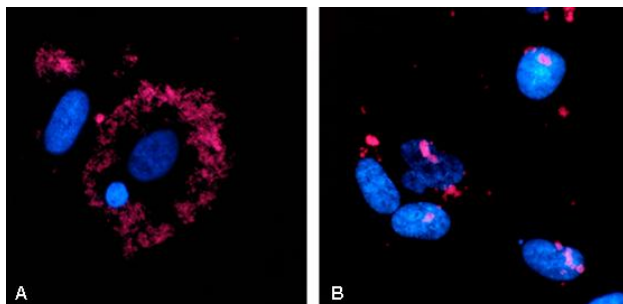
Figure 4. *DNA disposition (red) respect to cellular nucleus (blue) in ARPE-19 cells.* A: vector without protamine, B: vector with protamine.

Nevertheless, in HEK-293 cells DNA presented the same localization and the same condensation degree with both vectors (Figure 5), which means that the lack of lysosomal activity of via caveolae makes the release of protamine-DNA from the solid lipid nanoparticles difficult. As HEK-293 cells present a high division-rate and caveolae-mediated endocytosis localizes DNA close to the nucleus, use of protamine is not needed to transfect these cells and it becomes an obtacle because of its excesive DNA condesation.

Figure 5. *DNA disposition (red) respect to cellular nucleus (blue) in HEK-293 cells.* A: vector without protamine, B: vector with protamine.
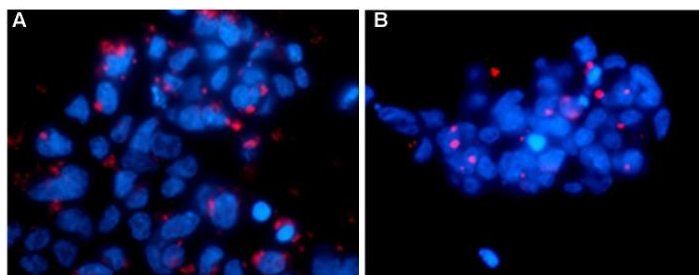
2. ELABORATION OF THE FINAL VECTOR BY MEANS OF ADDITION OF DEXTRAN: DEXTRAN-PROTAMINE-DNA-SLN VECTOR.

Having proved the need of lysosomal activity for the efficient working of vector bearing protamine, dextran was incorporated due to its typical use as clathrin-mediated endocytosis and endolysosomal marker, and because it would enhance this

cellular uptake mechanism.[34] Moreover, as previously mentioned, dextran is a safety and biocompatible anionic polysaccharide that has been used as a non-viral vector in many ways such as DEAE-dextran.[35]

The vector was prepared in a two-step procedure. Initially, a complex with dextran, protamine and DNA was prepared; negative charges of dextran and DNA bind to positive charges of the protamine. Then, SLN were added to complexes which bind by adsorption to nanoparticle surface due to interaction between free negative charges of complexes and cationic lipid present in SLN.[36] Three vectors of dextran-protamine-DNA-SLN at ratios: 0.5:2:1:5, 1:2:1:5 and 2:2:1:5 were prepared, and all of them showed capacity to protect DNA against DNAse and to release the DNA. Furthermore, the presence of dextran in the formulations did not result in statistically significant differences in size or zeta potential (p>0.05).

Transfection studies showed that presence of dextran significantly increased transfection of SLN in ARPE-19 cells. The most efficient formulation revealed a dextran-protamine-DNA-SLN ratio of 1:2:1:5. Moreover, it also increased cell viability, regarding control formulation. These data point to the importance of adequately optimizing the proportion of additives to prepare safe and efficient formulations. It is important to highlight the high transfection level obtained in ARPE-19 cells, close to 50% of cells transfected. This is even more intriguing, since these cells are difficult to transfect. For example, Abul-Hassan et al.[37] reported 25%

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[34] Agarwal A, Gupta U, Asthana A, Jain NK. Dextran conjugated dedritic nanoconstructs as potential vectors for anti-cancer agent. Biomaterials. 2009;30:3588-3596.

[35] Onishi Y, Eshita Y, Murashita A, Mizuno M, Yoshida J. Characteristic of DEAE-dextran-MMA graft copolymer as a nonviral gene carrier. Nanomedicine. 2007;3:184-191.

[36] Eliyahu H, Siani S, Azzam T, Domb AJ, Barenholz Y. Relationship between cehemical composition, physical propertis and transfection efficiency of polysaccharide-spermine conjugates. Biomaterials. 2006;27:1646-1655.

[37] Abul-Hassan K, Walmsley R, Boulton M. Opitimization of non-viral gene transfer to human primary retinal pigment cells. Curr Eye Res. 2000;20:361-366.

transfection efficiency for this cell line and other authors achieved even lower transfection rates.[20] Thus, the addition of dextran almost doubled the transfection levels achieved with SLN combined with protamine.

Regarding transfection capacity, HEK-293 cells were efficiently transfected only by DNA-SLN that reached around 60% percentage of transfected cells, whereas transfection obtained by the dextran-protamine-DNA-SLN (1:2:1:5) vector was 2.23%. These levels are according to EGFP production, since both results were 24-fold higher when the DNA-SLN vector was used (p<0.01). Since lipid particles are physiologically degraded, we followed transfection during 7 days to discard that delivery of the DNA in the cytoplasm might be dependent on time. Seven days after the addition of vectors, transfection levels did not increase in any of the two cell lines.

The addition of dextran and protamine did not modify the capacity of the vector to enter into the ARPE-19 cells and it decreased slightly the entry of vector in HEK-293, although, it was not enough to justify the great drop of transfection levels in this cell line. However the importance of the pathway involved in the cell uptake process was demonstrated again. As mentioned above, one of the reasons for the addition of dextran to our vector is the common use of dextrans as clathrin and endolysosomal markers. As expected, the presence of dextran induced a great internalization via clathrin of the dextran-protamine-DNA-SLN vector (R = 0.780 ± 0.050) in ARPE-19 cells that promoted a rise of transfection levels. Therefore, our previous observations have been confirmed here: indeed, the higher the entry via clathrin, the higher the transfection achieved with the vector bearing protamine is. Nevertheless, the low activity of clathrin-mediated endocytosis in HEK-293 cells diminishes the efficacy of the vector bearing dextran and protamine in this cell line (Box 7).
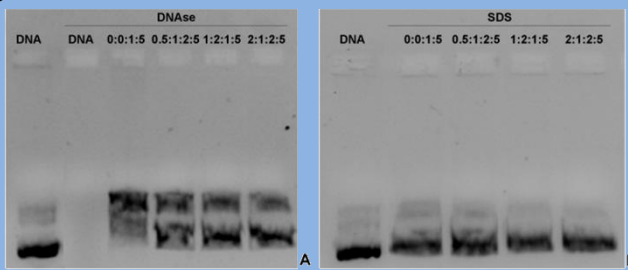
The presence of bands indicates that all formulations are able to protect DNA against DNAse (A) and release it from vectors (B).

	DNA-SLN 0:0:1:5	dextran-protamine-DNA-SLN 1:2:1:5
Size (nm)	283.63 ± 10.68	284.23 ± 11.87
Zeta potential (mV)	44.35 ± 6.62	40.60 ± 6.47
Polydispersion Index	0.42 ± 0.03	0.26 ± 0.02

Incorporation of dextran did not modify size or zeta potential.

Transfection levels in ARPE-19 cells				
dextran:protamine:DNA:SLN	Subset for alpha = 0.05 (Student-Newman-Keuls)			
	1	2	3	4
0:0:1:5	4.283			
0:2:1:5		28.880		
0.5:2:1:5		29.513		
1:2:1:5				48.016
2:2:1:5			34.486	
	% EGFP positive cells			

EGFP in ARPE-19 cells

DNA:SLN (1:5)   dextran:protamine:DNA:SLN (1:2:1:5)
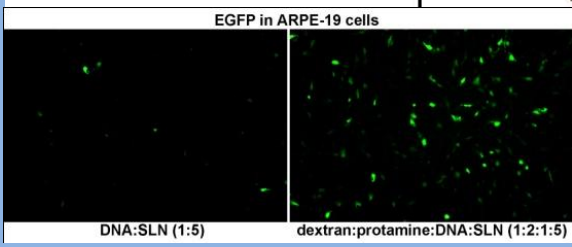
Formulations classified depending on the statistically significant differences of their transfection levels (at $p < 0.05$).
The best result was achieved by dextran-protamine-DNA-SLN vector (1:2:1:5).
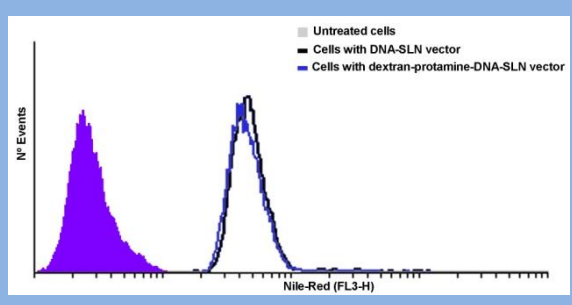
Dextran and protamine did not modify the capacity of SLN to enter into the ARPE-19 cells.

Presence of dextran induced a great internalization via clathrin of vectors that promoted a rise of transfection levels in ARPE-19 cells.

Colocalization via clathrin

Dex-Prot-DNA-SLN | Transferrin (Clathrin) | Colocalization

Colocalization via caveolae

Dex-Prot-DNA-SLN | Cholera (Caveolae) | Colocalization

Red: vector. Green: endocytosis marker.

ARPE-19 Cells

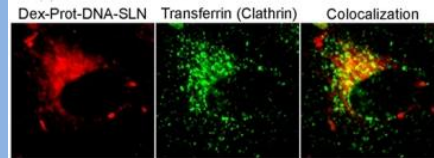|	Endocytosis Via Caveolae		Endocytosis Via Clathrin	
	DNA-SLN	Dex-Prot-DNA-SLN	DNA-SLN	Dex-Prot-DNA-SLN
R	0.535 ∓ 0.174	0.378 ∓ 0.037	0.424 ∓ 0.124	0.780 ∓ 0.050

R = Mander's Overloap coefficient; values from 1 (high colocalization) to 0 (low colocalization)

Transfection levels in HEK-293 cells

EGFP in HEK-293 cells

DNA:SLN (1:5) dextran:protamine:DNA:SLN (1:2:1:5)

HEK-293 cells were efficiently transfected only by DNA-SLN. ** $p<0.01$

Untreated cells
Cells with DNA-SLN vector
Cells with dextran-protamine-DNA-SLN vector

Dextran and protamine decreased lightly the capacity of SLN to enter into the HEK-293 cells.
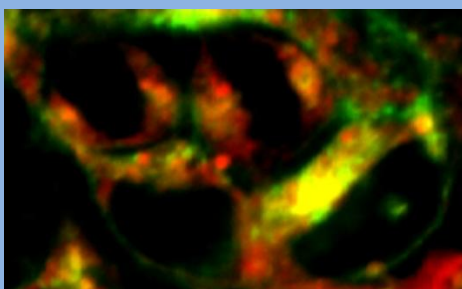
The high activity of caveolae-mediated endocytosis in HEK-293 cells favors the efficacy of DNA-SLN vector, and hampers the action of dextran-protamine-DNA-SLN vector, which needs the presence of via clathrin to work.

Red: DNA-SLN vector.
Green: caveolae marker.

Box 7. *Addition of dextran to vectors.*

3. "IN VITRO" STUDY OF THE DEXTRAN-PROTAMINE-DNA-SLN VECTOR WITH A THERAPEUTIC GENE.

Having developed an effective non-viral delivery system to transfect retinal pigment epithelial cells, pCMS-EGFP was replaced with a therapeutic plasmid, pCEP4-RS1, in order to confirm the utility of the vectors for X linked juvenile retinoschisis (XLRS) treatment. XLRS is a common cause of juvenile blindness in males with a prevalence of 1:5,000 to 1:25,000.[38] The application of gene replacement therapy in XLRS has been considered a promising therapeutic approach for this disease. Proof-of-concept was provided by several groups using viral delivery systems in retinoschisin-deficient mice.[39,40] However, viral vectors present important limitations due to immunogenicity and oncogenicity.[41] Moreover, there is evidence for the potential persistence of viral vectors in the brain after intravitreal injection.[42] These drawbacks have motivated the development of non-viral delivery systems.

Box 8 shows that a higher DNA:SLN ratio was necessary for pCEP4-RS1 than for pCMS-EGFP to maintain the protection capacity of the genetic material without affecting the DNA release ability (1:5 vs 1:6 ratios) since pCEP4-RS1 has a bigger molecular weight (11.1 Kb) than pCMS-EGFP (5.5 Kb). This plasmid codifies retisnoschin, an extracellular protein secreted by photoreceptors and bipolar cells that

[38] Mooy M, Van Den Born LI, Baarsma S, Paridaens DA, Kraaijenbrik T, Bergen A, et al. Hereitary X-linked juvenile retinoschisis: a review of the role of Müller cells. Arch Ophtalmol. 2002;120:979-984.

[39] Janssen A, Min SH, Molday LL, Tanimoto N, Seeliger MW, Hauswirth WW, et al. Effect of late-stage therapy on disease progression in AAV-mediated rescue of photoreceptor cells in retinoschisin-deficient mouse. Mol Ther. 2008;16;1010-1017.

[40] Min SH, Molday LL, Seeliger MW, Dinculescu A, Timmers AM, Janssen A, et al. Prolonged recovery of retinal structure/function after gene therapy in an Rs1h-deficient mouse model of x-loinked juvenile retinoschisis. Mol Ther. 2005;12:644-651.

[41] Kumar-Singh. Barrieres for retinal gene therapy: separating fact from fiction. Vision Res. 2008;48:1671-1680.

[42] Provost N, Le Meur G, Weber M, Mendes-Madeira A, Povedin G, Cherel Y, et al. Biodistribution of rAAV vectors following intraocular dministration: evidence for the presence and presistence of vector DNA in the optic nerver and in the brain. Mol Ther. 2005;11:275-583.

is involved in retinal organization and stability. Thus, cytoplasmatic retinoschisin was detected by immunochemistry and also levels of secreted retinoschisin were quantified (Box 8).[43] Fluorescence microscopy images depict that the vectors we prepared (SLN with and without protamine and dextran) were able to produce retinoschisin in the cytoplasm of ARPE-19 cells. Moreover, the protein secreted by cells was higher with the formulation containing protamine and dextran. These results are in line with the transfection results of pCMS-EGFP and prove the potential utility of the solid lipid nanoparticles based non-viral vectors loading XLRS1 plasmid.

When ratios DNA:SLN 1:5 and ratio dextran:protamine:DNA:SLN 1:2:1:5 were used, DNA was not totally bound to DNA-SLN vector and it was not totally protected. All of these drawbacks were overcome by using ratio DNA:SLN 1:6 and ratio dextran:protamine:DNA:SLN 1:2:1:6; release was not affected.
Red: DNA
Green: DNA-SLN vector
Blue: dextran-protamine-DNA-SLN vector.

ARPE-19 cells transfected with XLRS1 plasmid. The amount of retinoschisin (µg) per ml secreted by ARPE-19 cells treated with DNA-SLN vector was set to 100%

Box 8. *Vectors loaded with XLRS1 plasmid.*

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[43] Dyka FM, Wu WHW, Pfeifer TA, Molday LL, Grigliatti TA, Molday RS. Characterization and purification of the discoidin domain containing protein retinoschisin and its interaction with galactose. Biochemistry. 2008;47:9098-9106.

4. LOCAL APPLICATION OF THE DEXTRAN-PROTAMINE-DNA-SLN VECTOR BY DIFFERENT OCULAR ROUTES INTO RAT EYE.

After demonstrating the efficacy "in vitro" of dextran-protamine-DNA-SLN vector, a further preliminary "in vivo" study was carried out to assess whether the vectors are able to transfect ocular tissues. Generally, the eye is a promising target for gene therapy due to its unique features such as easy accessibility as well as convenient and highly sensitive methods for monitoring even minor changes of visual function. The well-defined anatomy and immunoprivilege of the eye are also important advantages for gene therapy.[44] Due to its relative small size, effective treatment of the ocular tissues will require minor product concentrations while the diffusion from the eye into the circulation is limited.[45,46]
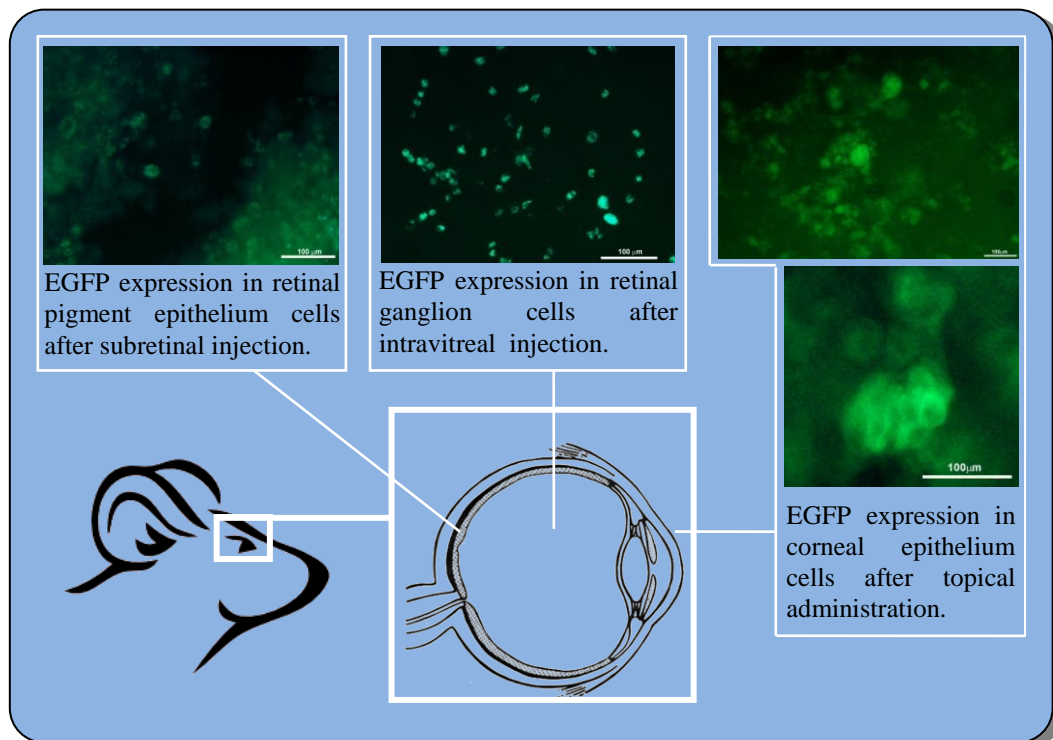
A dextran-protamine-DNA-SLN vector was loaded with a plasmid encoding enhanced green fluorescent protein (pCMS-EGFP) and injected into the eyes of Wistar rats by different administration routes: intravitreous, subretinal and topical. Expression of the green fluorescent protein was detected 72 hours after intravitreous and subretinal administration in various types of cells depending on the administration route (Box 9). There was a good response in retina ganglion cells when intravitreal injection was employed, but protein expression was poor in RPE cells. In contrast, after subretinal injection, the vector was able to transfect RPE cells as well as photoreceptors. These results are in line with those obtained in a previous study in which intravitreous administration of adenoviral vectors induced protein

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[44] Liu X, Brandt CR, Rasmussen CA, Kaufman PL. Ocular drug delivery: molecules, cells and gene. Can J Ophtalmol. 2007;42:447-454.

[45] Bloquel C, Bourges JL, Touchard E, Berdugo M, BenEzra D, Behar-Cohen F. Non-viral ocular gene therapy: Potential ocular therapeutic avenues. Adv Drug Deliv Rev 2006;58:1224-1242.

[46] Naik R, Mukhopadhyay A, Ganguli M. Gene delivery to the retina: focus on non-viral approaches. Drug Discov Today. 2009;14:306-315.

expression in the inner retina, whereas RPE cells and outer retina were the main transfected cells after subretinal administration.[47] This preliminary study may be useful to select the target cells to be transfected depending on the target of gene therapy. Non-viral vectors could be employed to treat different diseases such as glaucoma when the inner retina needs to be addressed, or retinoschisis and retinitis pigmentosa, if administration reaches the outer retina.[48,49]

EGFP expression in retinal pigment epithelium cells after subretinal injection.

EGFP expression in retinal ganglion cells after intravitreal injection.

EGFP expression in corneal epithelium cells after topical administration.

Box 9. *Transfection achieved in eye by dextran-protamine-DNA-SLN vector.*

[47] Colella P. Cotugno G, Auricchio A. Ocular gene therapy: current progress and future prospects. Trens Mol Med. 2009;15:23-31.

[48] Johnson EC, Guo Y, Cepurna WO, Morrison JC. Neurotrophin roles in retinal ganglion cell survival: Lessons from rat glaucoma models. Exp Eye Res. 2009;88:808-815.

[49] Phelan JK, Bok D. A brief review of retinitis pigmentosa and the indentified retinits pigmentosa gene. Mol Vis. 2000;6:116-124.

The vector was also able to transfect corneal cells after topical application (Box 9). As this administration is a non-invasive route, and cornea immune defense does not induce inflammation, this vector may be a safe method to treat corneal endothelial dystrophies and to modulate protein production in order to control the corneal microenvironment;[50] all this, without the possible limitations that viral vectors present in ocular gene therapy.

Taken together, this data provides evidence for the potential application of the dextran-protamine-DNA-SLN vector for the treatment of degenerative retina disorders as well as ocular surface diseases.

5. SYSTEMIC RESPONSE OF THE DEXTRAN-PROTAMINE-DNA-SLN VECTOR AFTER INTRAVENOUS ADMINISTRATION.

The good response obtained by the dextran-protamine-DNA-SLN vector after local application in rat eye led us to prove the capacity of this vector to induce protein expression after intravenous injection to mice. After intravenous administration of genetic material, several obstacles such as digestion by nucleases and hepatic uptake clearance must be overcome (Figure 6). Although these drawbacks could be solved by using gene delivery systems,[51] a handicap related to the positive charge of some non-viral vectors has to be considered. Positive charges of cationic lipids may interact with other lipid particles, serum proteins and erythrocytes that could lead to aggregations in the bloodstream and hemagglutination[52] that may have influence on

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[50] Klausner EA, Peer D, Chapman RL, Multack RF, Andurkar SV. Corneal gene therapy. J Control Release. 2007;124:107-133.

[51] Liu F, Shollenberg LM, Conwell CC, Yuan X, Huang L. Mechanism of naked DNA clearance after intravenous injection. J Gene Med. 2007;9:613-619.

[52] Eliyahu H, Servel N, Domb AJ, Barenholz Y. Lipoplex-induced hemagglutination: potential involvment in intravenous gene delivery. Gene Ther. 2002;9:850-858.

transfection, especially "in vivo".[16] Therefore, the addition of dextran, an anionic biocompatible polysaccharide, as third component to our vector should minimize these factors related to toxicity.
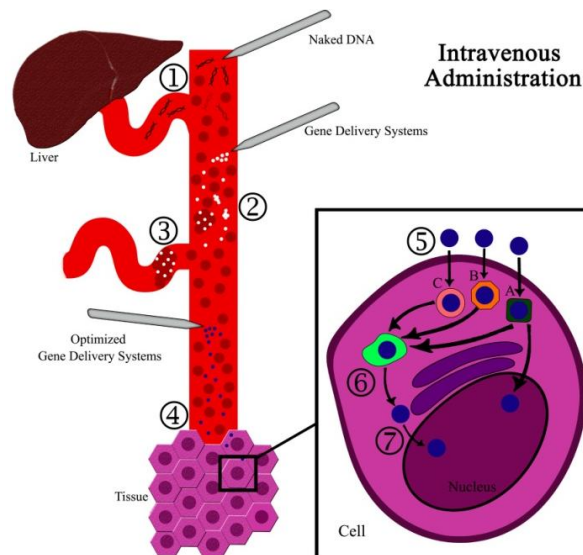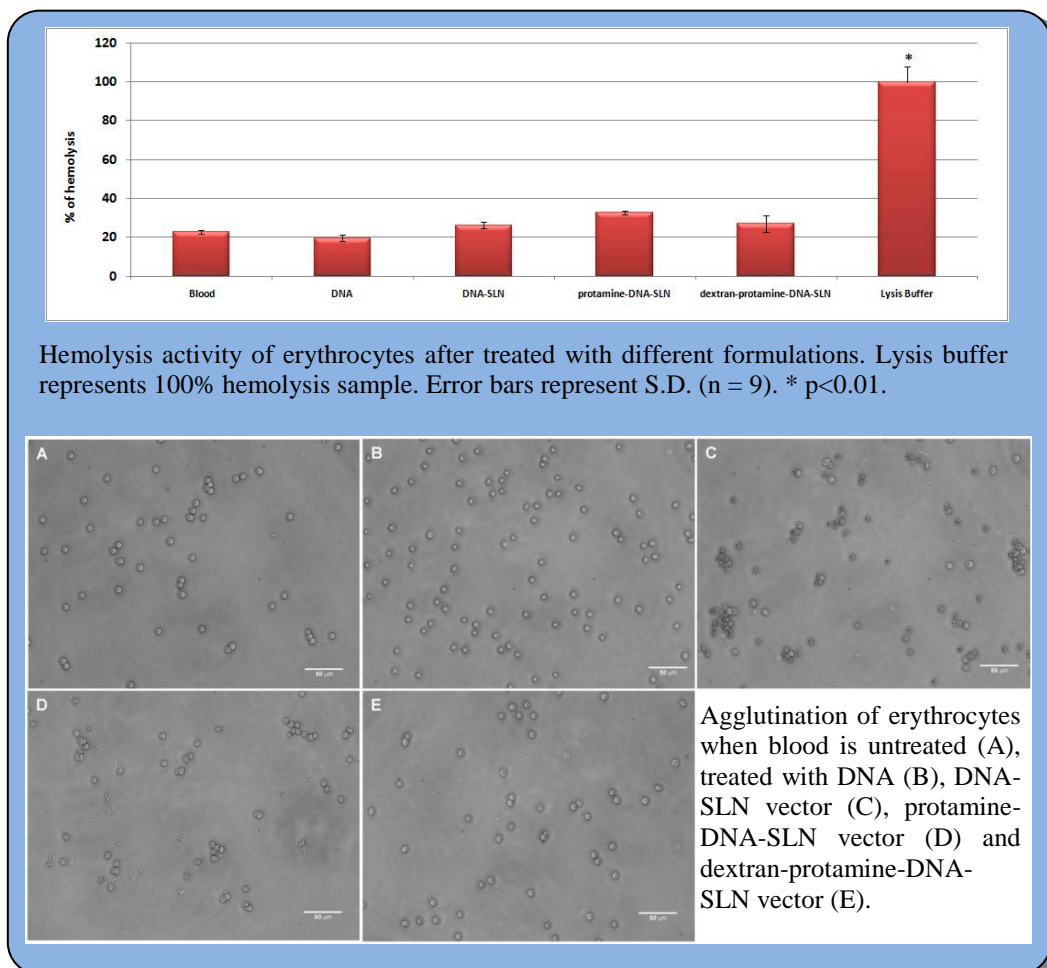

Figure 6. Use of gene delivery systems is necessary to administrate DNA by intravenous route since it is removed quickly by nucleases and hepatic clearance (1). However, positive charges of non-viral vectors could cause aggregations (2) and hemagglutination (3) that will have influence on efficacy and toxicity. Thus, it is essential optimize gene carriers to solve those problems and overcome the intracellular barriers. Vector has to facilitate the pass through cell membrane (5) and it can modulate the uptake mechanisms such as endocytosis via caveolae (A), via clathrin (B) or clathrin/caveolae-independent endocytosis (C). Promoting endosomal scape (6) is also important to improve the efficacy, as well as the translocation through nuclear membrane (7).

As mentioned above, the addition of dextran and protamine did not modify the size and superficial charge in comparison with the DNA-SLN vector. Protamine and dextran are positively and negatively charged, respectively, and a compensation of charges may occur.

Despite the lack of significant differences in size and superficial charge, differences in the interaction with erythrocytes between the formulations were

detected. The DNA-SLN vector showed low hemagglutination capacity, whereas the dextran-protamine-DNA-SLN vector showed no agglutination. We also detected hemagglutination with a formulation consisting of protamine, DNA and SLN at 2:1:5 ratio. This indicates that the lack of hemagglutination effect of the dextran-protamine-DNA-SLN is due to the presence of dextran on the nanoparticle surface. Besides hemagglutination, hemolytic activity, which is also indicative of cytotoxicity, was negligible with all the formulations (Box 10).

Hemolysis activity of erythrocytes after treated with different formulations. Lysis buffer represents 100% hemolysis sample. Error bars represent S.D. (n = 9). * p<0.01.

Agglutination of erythrocytes when blood is untreated (A), treated with DNA (B), DNA-SLN vector (C), protamine-DNA-SLN vector (D) and dextran-protamine-DNA-SLN vector (E).

Box 10. *Interaction of vectors with erythrocytes.*

After intravenous administration to mice, lipid nanoparticles with protamine and dextran managed to obtain a significant response in liver, spleen and lung. Three days after the administration of the formulation the percentage of positive sections (presence of EGFP) was 70% in lung, 85% in liver and 100% in spleen. Transfection was still detected in the three organs 7 days post-administration, and it even increased in lung (Figure 7). The DNA-SLN formulation, previously assayed in mice by intravenous route, also showed transfection in liver and spleen but not in lung. Moreover, protein expression decreased greatly 7 days after administration.[22]
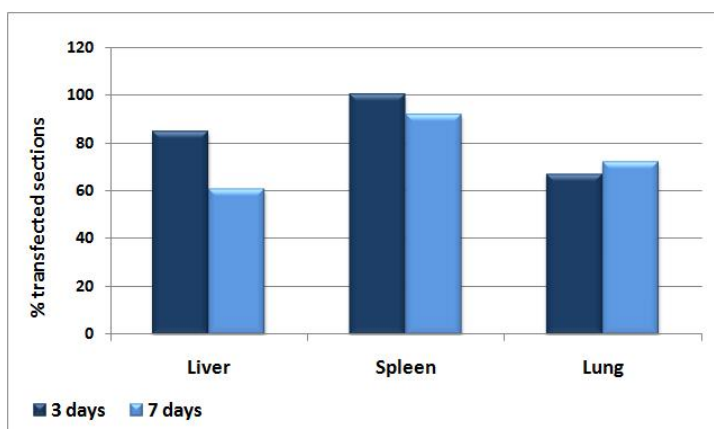

Figure 7. Percentage of transfected sections of liver, spleen and lung 3 and 7 days after treatement with the dextran-protamine-DNA-SLN vector. The SLN to DNA ratio (w/w) was 5:1 and the dextran-protamine to DNA ratio 1:2:1 (w/w/w).

The increase of transfection over time by the vector containing protamine and dextran with respect to the DNA-SLN vector suggests a longer circulation time.[53,54] The "in vitro" study revealed that dextran was able to avoid the erythrocytes aggregation and, therefore, the longer circulation stay in the bloodstream may be due to the presence of dextran on the nanoparticle surface. The high DNA condensation of protamine that enhances the nuclease resistance may also result in an extended stay of plasmid in the organism. This fact could be also involved in the transfection obtained

[53] Chrastina A, Keri AM, Schnitzer JE. Overcoming *"in vivo"* barriers to targeted nanodelivery. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2011;3:421-437.

[54] Fischer D, Osburg B, Petersen H, Kissel T, Bickel U. Effect of poly(ethylene imine) molecular weigth and pegylation on organ distribution and pharmacokinetics of polyplex with oligodeoxynucleotides in mice. Drug Metab Dispos. 2004;32:983-992.

in lung. Moreover, the presence of NLS in protamine, which improves the nuclear envelope translocation and its capacity to facilitate transcription,[27] may also improve the transfection efficacy "in vivo".
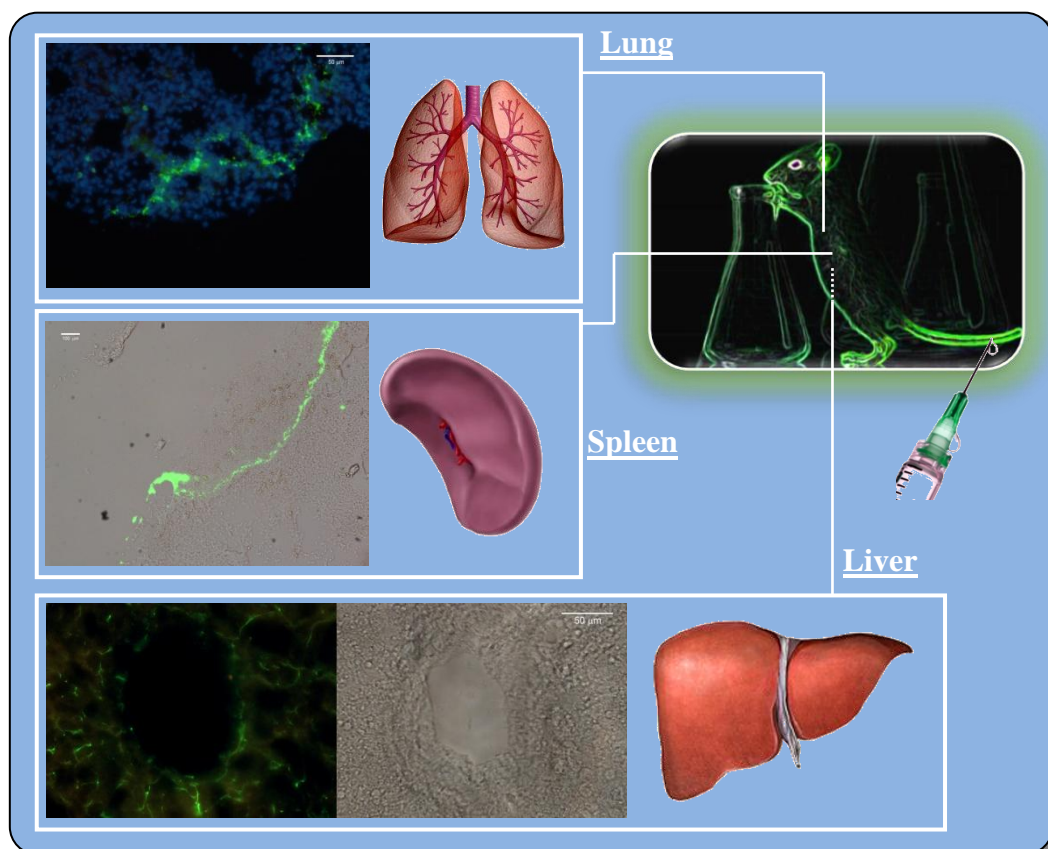
Box 11. *Transfection achieved after intravenous administration.*

The anatomical and physiological characteristics of liver and spleen, more specifically the discontinuous endothelium, facilitate the uptake of the nanoparticles. This contributes to efficiently transfect these organs. These results suggest the potential usefulness of this vector when liver and spleen are the target organs,[53] for example in the treatment of diseases such as liver or spleen tumors,[55] hepatitis B[56] or

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[55] Tang H, Tang XY, Liu M, Li X. Targeting alpha-fetoprotein represses the proliferation of hepatoma cells via regulation of the cell cycle. Clin Chim Acta. 2008;394:81-88.

for the design of DNA vaccines.[57] The liver could also be used as a depot organ to produce large amounts of a therapeutic enzyme that is secreted to the bloodstream and recaptured by other organs.[58] The ability of our new vector to transfect lung makes it useful as a potential system for the treatment of diseases such as acute respiratory distress syndrome (ARDS), cancer, asthma, emphysema and cystic fibrosis (CF).[59] Although these are clearly promising results, additional studies would be needed to assess the real potential of this new vector.

Over the past few years, the development of carriers to target caveolae-mediated endocytosis is emerging as a strategy to improve non-viral vectors.[30,53,60] We have developed a new gene delivery system that, when internalized by caveolae/raft-mediated endocytosis, was not able to transfect "in vitro", but managed a better response "in vivo" than the DNA-SLN vector, which was also internalized by caveolae/raft-mediated endocytosis and transfected efficiently "in vitro". This lack of "in vitro"-"in vivo" correlation, accepted for this kind of formulations, jointly with the lack of adequate methods to better understand the process of endocytosis and intracellular trafficking of gene carriers "in vivo",[30] justifies the need of studies with animal models during the early stages of the development of gene delivery systems.

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[56] Zhang Y, Rong Qi X, Gao Y, Wei L, Maitani Y, Nagai T. Mechanism of co-modified liver targeting liposomes as gene delivery carrieres base don celular uptake and antigens inhibition effect. J Control Release. 2007;117:281-290.

[57] Raska M, Moldoveanu Z, Novak J, Hel Z, Bozja J, Compans RW, et al. Delivery of DNA HIV-1 vaccine to the liver induces high and long-lasting humoral inmune responses. Vaccine. 2008;26:1541-1551.

[58] Mango RL, Xu L, Sands MS, Vogler C, Seiler G, Schwarz T, et al. Neonatal retroviral vector-mediated hepatic gene therapy reduces bone, joint and cartilage diseases in mucopolysacchatidosis VII mice and dogs. Mol Genet Metab. 2004:82:4-19.

[59] Griesenbach U, Alton EW. Cystic fibrosis gene therapy: succeses, failures and hopes for the feature. Expert Rev Respir Med. 2009;3:363-371.

[60] Liu C, Yu W, Chen Z, Zhang J, Zhang N. Enhanced gene transfection efficiency in CD13-positive vascular endothelial cells with targeted poly (lactic acid) –poly (ethylene glycol) nanoparticles through caveolae-mediated endocytosis. J Control Release. 2011;151:162-195.
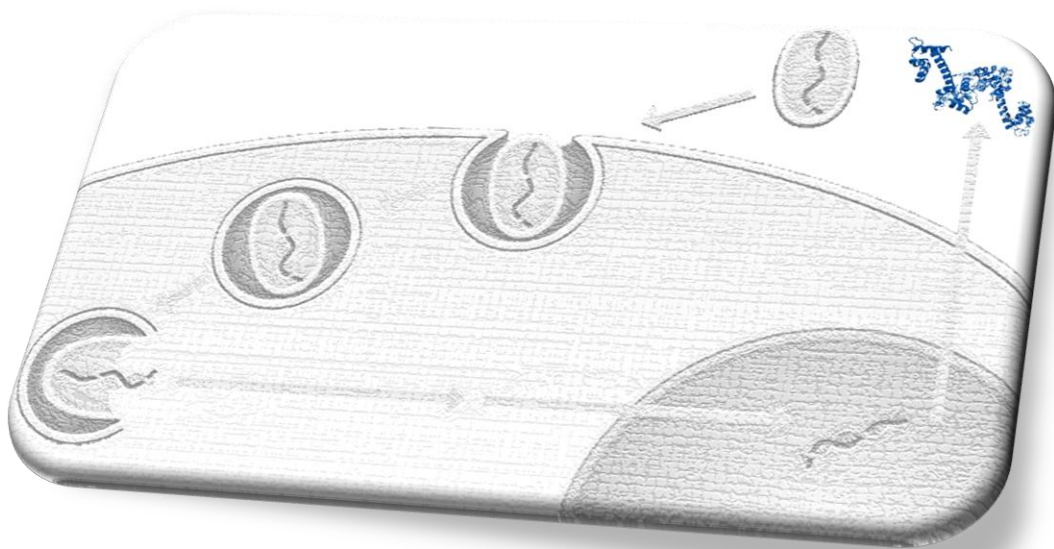
CONCLUSIONS

From the results obtained in the experimental work reported on here we have derived the following conclusions:

1. Precondensation of DNA with protamine and its incorporation to solid lipid nanoparticles increases its protection against DNases and favors the intracellular diffusion and its approach to the nucleus.

2. Protamine induced a 6-fold increase in the "in vitro" transfection capacity of solid lipid nanoparticles in ARPE-19 cells. This is due to a higher DNA condensation, the presence of Nuclear Localization Signals in protamine and a shift in the internalization mechanism from caveolae/raft-mediated endocytosis to clathrin-mediated endocytosis. Because of its lisosomal activity, clathrin-mediated endocytosis would favor the protamine-DNA complex release from the nanoparticles and, consequently, transfection.

3. Protamine diminished the "in vitro" transfection capacity of solid lipid nanoparticles in HEK-293 cells. On the one hand, the presence of protamine decreases the cell uptake of the vectors. On the other hand, the low activity of clathrin-mediated endocytosis in comparison with caveolae/raft-mediated endocytosis in this cell line causes a greater entry of vectors by caveolae/raft-mediated endocytosis. The low lisosomal activity would hamper the protamine-DNA complex release from the nanoparticles and, consequently, transfection.

4. The addition of dextran to protamine-DNA-SLN complexes favors the entry of the vector by means of clathrin-mediated endocytosis, achieving 44% of transfection efficacy "in vitro" in ARPE-19 cells.

5. The dextran-protamine-DNA-SLN vector loading XLRS1 plasmid manages to transfect "in vitro" ARPE-19 cells and to induce the expression of retinoschisin. These results prove the potential utility of this vector for the treatment of X linked juvenile retinoschisis and other retinal degenerative diseases by means of gene therapy.

6. After ocular administration to rats, the dextran-protamine-DNA-SLN vector induces the expression of a foreign protein, the green fluorescent protein, in various types of cells depending on the administration route. After intravitreal injection, retina ganglion cells are mainly transfected, whereas if the vector is administrated by subretinal injection, retinal pigment epithelium cells as well as photoreceptors are the most transfected cells. After topical application, the vector is also able to transfect corneal cells.

7. The dextran-protamine-DNA-SLN vector is able to induce the expression of a foreign protein, the green fluorescent protein, in the spleen, liver and lung after intravenous administration to mice; the expression was maintained for at least seven days.

De los resultados obtenidos en los trabajos experimentales de la presente memoria podemos extraer las siguientes conclusiones:

1. La precondensación del ADN con protamina y posterior incorporación en las nanopartículas sólidas lipídicas incrementa la protección frente a DNasas, favorece la difusión intracelular del material genético y su aproximación al núcleo.

2. La protamina incrementó seis veces la capacidad de transfección "in vitro" de las nanopartículas sólidas lipídicas en células ARPE-19. Este aumento se debe a la mayor condensación del ADN, a la presencia de señales de localización nuclear en la protamina y a un cambio en el mecanismo de internalización de endocitosis vía caveola a endocitosis vía clatrina. Debido a la actividad lisosomal, la endocitosis vía clatrina, favorecería la liberación del complejo protamina-ADN de la nanopartícula y por tanto, la transfección.

3. La protamina disminuyó la transfección "in vitro" de las nanopartículas sólidas lipídicas en las células HEK-293. Por un lado, la presencia de protamina hace que disminuya la captación celular. Por otro lado, la baja actividad de la endocitosis vía clatrina en estas células hace que las nanopartículas entren mayoritariamente por endocitosis vía caveola. La menor actividad lisosomal dificultaría la liberación del complejo protamina-ADN de la nanopartícula y por tanto, la transfección.

4. La adición de dextrano a los complejos protamina-ADN-SLN favorece la entrada del vector por endocitosis vía clatrina, alcanzándose una eficiencia de transfección "in vitro" en las células ARPE-19 del 44%.

5. El vector dextrano-protamina-ADN-SLN con el plásmido XLRS1 es capaz de transfectar "in vitro" células ARPE-19 e inducir la expresión de la retinosquisina. Estos resultados demuestran la potencial utilidad de este vector en el tratamiento de la retinosquisis juvenil ligada al sexo y otras enfermedades degenerativas de la retina mediante terapia génica.

6. Tras su administración ocular en ratas, el vector dextrano-protamina-ADN-SLN induce la expresión de una proteína exógena, la proteína verde fluorescente, en varios tipos celulares dependiendo de la vía de administración. Tras la administración intravítrea, se transfectan mayoritariamente las células ganglionares, mientras que cuando el vector se administra por vía subretiniana, las células que más se transfectan son las células del epitelio pigmentario de retina y los fotorreceptores. Tras la administración por vía tópica, el vector es capaz de transfectar células corneales.

7. El vector dextrano-protamina-ADN-SLN es capaz de inducir la expresión de una proteína exógena, la proteína verde fluorescente, en bazo, hígado y pulmón tras su administración intravenosa en ratones, manteniéndose la expresión durante al menos siete días.

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